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14. ABSTRACT

Shiga toxin (Stx) types 1 and 2 are encoded within intact or defective temperate bacteriophages in Stx-producing Escherichia coli (STEC), and expression of these toxins is linked to bacteriophage induction. Among Stx2 variants, only stx2e from one human STEC isolate has been reported to be encoded within a toxin-converting phage. In this study, I examined O91:H21 STEC isolate B2F1 that carries two functional alleles (stx2d1 and stx2d2) for the potent activatable Stx2 variant toxin, Stx2d, for the presence of Stx2d converting bacteriophages and other potential regulators of toxin expression. Mutants of B2F1 that produced one or the other Stx2d toxin were made. The Stx2d1-producing mutant (stx2d2::cat) was less cytotoxic for Vero cells than the Stx2d2-producing mutant (stx2d1::cat). Consistent with those results, the Stx2d1-producing mutant was attenuated in a streptomycin-treated mouse model of STEC infection, while the Stx2d2-producing mutant was nearly as virulent as wild-type B2F1. When the mutants were treated with mitomycin C to promote bacteriophage induction, Vero cell cytotoxicity was elevated only in extracts of the Stx2d1-producing mutant. Additionally, when mice were treated with ciprofloxacin, an antibiotic that induces the O157:H7 Stx2-converting phage, the animals were more susceptible to the Stx2d1-producing mutant. An stx2d1-containing lysogen was isolated from plaques on strain DH5α that had been exposed to lysates of the mutant that produced Stx2d1 only. However, that RecA- lysogen could not be induced for phage nor were culture lysates from it cytotoxic for Vero cells. By contrast, when the lysogen was transformed with a plasmid encoding RecA and induced with mitomycin C, culture extracts were cytotoxic for Vero cells. Furthermore, electron microscopic examination of extracts from the ϕB2F1-lysogen showed hexagonal particles that resembled the prototypic Stx2-converting phage 933W. These observations provide strong evidence that expression of Stx2d1 is bacteriophage-associated. The finding that synthesis of Stx2d1 but not Stx2d2 was associated with phage induction led me to investigate regulation of Stx2d2 production. Transposon mutagenesis of DH5α revealed genes associated with reduced expression of an stx2d2 promoter::lacZ fusion in a reporter plasmid, observations that suggested the inactivation of potential activators of transcription of stx2d2. The mutant genes were isolated and sequenced. Two mutations identified were in caiD and the div gene of DH5α; these mutants showed reduced cytotoxicity and virulence for mice when transformed with an Stx2d2 toxin gene clone as compared to wild-type DH5α similarly transformed. However, introduction of the same mutations into strain B2F1 did not result in reduced cytotoxicity or virulence. The influence of the caiD and div gene mutations on toxin expression in DH5α may be polar and not correspond to the arrangement of these genes in B2F1. Alternatively, the effects of these mutations may only be evident when multiple copies of the toxin gene are present. In sum, the regulation of expression of the two Stx2d alleles in B2F1 differs such that Stx2d1 expression is tightly repressed except under conditions that induce the toxin-converting phage in which it is encoded. In contrast, Stx2d2 is expressed at higher levels, independent of phage induction, under the direction of some other yet-to-bedefined host factor. The DNA sequences that flank stx2d2 share extensive homology to those flanking stx2d1 and other phage-borne Stx genes, a finding that suggests that stx2d1 and stx2d2 share a common phage origin but the phage sequences associated with stx2d2 are defective.

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ABSTRACT

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The Regulation of Expression of the Stx2d Toxins in Shiga Toxin-producing *Escherichia coli* O91:H21 Strain B2F1

Louise D. Teel, Doctor of Philosophy, 2002

Thesis directed by:

Alison D. O'Brien, Ph.D.

Professor and Chair, Department of Microbiology and Immunology

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The Regulation of Expression of the Stx2d Toxins in Shiga Toxin-producing *Escherichia coli* O91:H21 Strain B2F1

by

Louise Davis Teel

Dissertation submitted to the Faculty of the

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To Barbara Florence Mateer Davis

for her faith,

for her generosity,

for her devotion to education,

for her commitment to civil rights and feminism,

and for her undying ability to find so much in life hilarious.

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INTRODUCTION

I. Shiga toxin-producing *Escherichia coli* (STEC)

A. History. In 1982, *Escherichia coli* serotype O157:H7 was linked with two small outbreaks of acute bloody diarrhea in Oregon and Michigan. These cases of illness were associated with the consumption of ground beef (Riley *et al.*, 1983). O'Brien *et al.* showed that extracts of a culture of the *E. coli* O157:H7 strain isolated from hamburger (CDC designation EDL933) were cytotoxic to Vero cells (African green monkey kidney cells). The cytotoxic effect of the culture extracts was neutralized with polyclonal antiserum raised against Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1. Because of this antigenic similarity between the toxin produced by *E. coli* O157:H7 and Shiga toxin, the term Shiga-like toxin was adopted (O'Brien *et al.*, 1983). In the same year in Canada, *E.coli* O157:H7 was isolated during an outbreak of hemorrhagic colitis (HC) in a nursing home. Bacteria from that outbreak produced Shiga-like toxin, also known as Verotoxin because of its effect on Vero cells (Johnson *et al.*, 1983).

In 1983, Karmali *et al.* reported the isolation of Verotoxin-producing *E.coli* from eight Canadian children with the hemolytic uremic syndrome (HUS) (Karmali *et al.*, 1983). HUS is a potentially life-threatening disease, the onset of which is usually preceded by a bout of bloody diarrhea. In a comprehensive study of children with HUS reported in 1985, the same investigators showed evidence of current or recent infection with Verotoxin-producing bacteria in 75% of the children with HUS (Karmali *et al.*, 1985).

By 1985, *Escherichia coli* capable of producing Shiga-like toxins (referred to from here on by their current designation Shiga toxin-producing *E. coli*) were associated with both HC and HUS. Characterization of the Shiga toxins (Stxs) produced by O157:H7 serotype *E. coli* later revealed that there were two types. One is essentially identical to Shiga toxin and the other is antigenically distinct but very closely related in structure and function to Shiga toxin (Strockbine *et al.*, 1988; Strockbine *et al.*, 1986; Takao *et al.*, 1988). The nomenclature of these *E. coli* toxins has been revised over time, and they are currently designated as Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2), respectively. In contrast to Shiga toxin in *S. dysenteriae*, Stx1 and Stx2 were shown to be bacteriophage-borne (see below) (O'Brien *et al.*, 1984; O'Brien *et al.*, 1989; Strockbine *et al.*, 1986; Scotland *et al.*, 1983; Smith *et al.*, 1983).

B. Epidemiology. The O157:H7 serotype was considered a rare *E. coli* serotype in the early 1980s, but its emergence as a significant pathogen in the United States has since been well established (Centers for Disease Control and Prevention, 1994). Laboratory identification of O157:H7 *E. coli* was facilitated by the advent of sorbitol MacConkey agar (SMAC) to distinguish sorbitol non-fermenting O157:H7 organisms from most normal fecal *E. coli* (Wells *et al.*, 1983). By 1987, O157:H7 was recognized to be more prevalent than *Shigella* in the United States and was designated the leading cause of hemorrhagic colitis in 1992 (Centers for Disease Control and Prevention, 1994). Now *E. coli* of that serotype are considered the major cause of hemolytic uremic syndrome and kidney failure in children (Banatvala *et al.*, 2001; Griffin and Tauxe, 1991) and are associated with a broad spectrum of diseases ranging from mild non-bloody diarrhea to HUS and thrombotic thrombocytopenic purpura (TTP) (Griffin *et al.*, 1988).

The O157:H7 serotype of STEC is the most common one in the United States and Canada, but many non-O157:H7 strains *E. coli* have also been associated with the diseases listed above. Mead *et al.*, of the Centers for Disease Control and Prevention (Mead *et al.*, 1999), recently estimated that 73,000 cases of O157:H7-associated diarrhea per year occur in the United States. Furthermore, these investigators estimated that 4% of the *E. coli* O157:H7-infected patients (which includes adults and children) will develop the hemolytic uremic syndrome. Mead and colleagues also predicted that an additional 36,000 non-O157:H7 cases of hemorrhagic colitis occur annually in the United States. The identification of non-O157:H7 serotypes of *E. coli* has been more difficult for the clinical laboratory since most such strains ferment sorbitol and are not readily detected with SMAC agar. In addition, these non-O157 STEC represent diverse serotypes; therefore, O antigen detection methods are not practical. Nevertheless, all of the serotypes associated with HC and HUS produce Shiga toxin, thus, toxin detection may be the most valuable clinical tool for identification.

The role of STEC as an emerging pathogen is not limited to the United States. STEC disease has been reported in Japan, Canada, South America, Great Britain, Europe, Australia and Africa, particularly in areas where cattle-rearing is economically important (Michino *et al.*, 1998; Griffin, 1998; Spika *et al.*, 1998; López *et al.*, 1998; Caprioli and Tozzi, 1998; Smith *et al.*, 1998; Robins-Browne *et al.*, 1998; Effler *et al.*, 2001). Cattle harbor STEC asymptomatically in the gastrointestinal tract, and humans become infected by ingestion of food or beverages contaminated with manure or by secondary transmission from infected individuals (Griffin and Tauxe, 1991; Armstrong *et al.*, 1996). In addition to inadequately cooked ground beef, many other foodstuffs have been

implicated in the transmission of O157:H7 *E. coli*. These food items include sprouts (Michino *et al.*, 1999; Breuer *et al.*, 2001), dairy products (MacDonald *et al.*, 1988), unpasteurized apple juice (Besser *et al.*, 1993), salad greens (Abdul-Raouf *et al.*, 1993), sausage (Ammon *et al.*, 1999) and well water (Jackson *et al.*, 1998).

The severity of disease associated with STEC infection is age related. Young children and elderly adults are the most likely to experience severe sequelae after infection. The risk of developing HUS is highest for children. Approximately 10% of infected children under age ten develop HUS (Tarr, 1995). HUS occurs in adults as well, but TTP is observed more often (Tarr, 1995; Banatvala *et al.*, 2001). Both HUS and TTP are marked by hemolytic anemia, thrombic thrombocytopenia, and renal dysfunction; however, TTP is generally associated with fever and neurological symptoms as well.

C. Definitions of Enterohemorrhagic *Escherichia coli* (EHEC) and STEC. The term STEC is based on the first described and most universal virulence property of *E. coli* associated with hemorrhagic colitis and hemolytic uremic syndrome, the production of Shiga toxin (O'Brien *et al.*, 1983; Johnson *et al.*, 1983; Karmali *et al.*, 1985). In addition to production of one or more types of Shiga toxin, O157:H7 *E. coli* has characteristics previously described in enteropathogenic *E. coli* (EPEC), a large 90 kilobase (kb) plasmid in STEC that may encode potential virulence-associated genes, and the capacity to produce attachment and effacement (A/E) lesions *in vivo* in the intestines of laboratory animals (Levine, 1998; Tzipori *et al.*, 1986; Tzipori *et al.*, 1987). Because all these factors may have a role in pathogenesis, Levine proposed the definition of Enterohemorrhagic *Escherichia coli* (EHEC) as those *E. coli* associated with hemorrhagic colitis that: i.) produce Shiga toxin; ii.) possess a large plasmid of

approximately 90 kb that is recognized by hybridization with a DNA probe specific for the plasmid-encoded hemolysin gene ; and, iii.) produce A/E lesions *in vivo*. This EHEC definition applies to O157:H7, O26:H11, and O111: NM (non-motile) serotypes. The latter two were previously classified as EPEC strains. Unfortunately, this definition does not include the numerous STEC that do not produce intimin, a protein essential to the generation of A/E lesions (see below), but are associated with HC and its sequelae. In sum, STEC are all *E. coli* that produce Stx1, Stx2 (or a variant thereof) or both, many strains of which also harbor a large plasmid. EHEC are an intimin positive, 90 kb plasmid positive subset of STEC.

Recently, STEC have been divided into four groups that reflect the evolutionary relatedness of several strains within each category (Whittam *et al.*, 1993). Whittam *et al.,* categorized these STEC by multilocus enzyme electrophoresis and assessed the extent of divergence of a battery of essential cellular enzymes from hundreds of strains and serotypes isolated worldwide. By these criteria, the EHEC 1 group consists only of O157:H7 and O157:NM, two serotypes that appear to represent recent clones that have spread globally. Members of the EHEC 1 group synthesize one or more types of Shiga toxin, contain the EHEC plasmid, and produce intimin. In addition, members of the EHEC 1 group share an enzyme profile that Whittam suggests evolved from an EPEC ancestor, O55:H7. The EHEC 2 group is characterized by the three EHEC properties defined above and includes serotypes O26:H11, O111:H8, and O111:H11 or NM. The enzyme profiles of these serotypes suggest a distinct origin from members of the EHEC 1 group.

The STEC 1 category contains a diverse group of serotypes that often share the same flagellar antigen, H21, and do not express intimin. Representatives of the STEC 1 group have been isolated from humans and bovine animals in North America, Europe and Asia. The strain used in the studies described in this dissertation, an O91:H21 strain B2F1, belongs to this group. The STEC 2 group contains O103:H2, O103:H6 and O45:H2. They are also intimin negative Shiga toxin-producers clonally distinct from STEC 1.

D. Virulence factors associated with EHEC and STEC strains.

1. Shiga toxin (Stx). Because of the risk of life-threatening sequelae associated with Shiga toxin, comparative human volunteer studies with toxin-producing strains have not been conducted. However, we know from naturally-occurring infections with *Shigella dysenteriae* that HUS is associated with the toxigenic strain type 1 (Butler *et al.*, 1987). Experimental infection in primates fed toxin-deletion mutants of *S. dysenteriae* type 1 resulted in decreased blood in the stool, less tissue damage and inflammation of the colonic mucosa, and reduced vasculitis in the colonic mesothelium (Fontaine *et al.*, 1988). A similar effect was seen in the rabbit model of diarrhea with the natural EPEC-like lapine pathogen RDEC-1. Rabbits infected with RDEC-1 lysogenized with the Stx1 toxin-converting phage showed enhanced inflammation, edema, and vascular changes associated with Stx1 production when compared with rabbits infected with RDEC-1 that did not produce Stx1 (Sjogren *et al.*, 1994). *In vitro* experiments showed that Shiga toxin is cytotoxic to cultured human intestinal cells (Moyer *et al.*, 1987) and causes fluid accumulation in the ligated rabbit ileal loop model (Keenan *et al.*,

1986). Together these findings provide strong evidence of the role of Shiga toxin in bloody diarrhea.

Evidence of the involvement of Shiga toxin in HUS is indirect. Shiga toxin binds the Gb_3 receptor found on vascular and renal endothelial tissue (Lingwood *et al.*, 1987). One hypothesis is that the binding of Shiga toxin to its receptor triggers thrombin activation and initiation of the coagulation cascade (Chandler *et al.*, 2002). Renal damage may follow from accumulation in the tubules and glomeruli of fibrin thrombi (Chandler *et al.*, 2002). In addition, Stx may have a direct effect on renal tissue. Indeed, Shiga toxin is toxic to renal cells *in vitro* (Obrig *et al.*, 1988; Tesh *et al.*, 1991) and has been shown by immunohistochemistry to be localized in renal tissue of O157:H7-associated HUS patients (Chaisri *et al.*, 2001). Systemic disease is generally more profound in children than adults, presumably due to greater toxin receptor expression in renal tissue of children (Chaisri *et al.*, 2001).

Typical HUS pathology has been observed in animal models with Stx exposure by different routes. In one such study, intravascular injection of Stx1 in rabbits resulted in the same type of microvascular angiopathy seen in the glomeruli of HUS victims (Richardson *et al.*, 1992). Ferrets infected orally with O157:H7 showed glomerular necrosis similar to that seen in humans with HUS (Woods *et al.*, 2002). In the mouse model of STEC infection in which mice are pre-treated with streptomycin, renal tubule necrosis was seen, in contrast to glomerular damage (Wadolkowski *et al.*, 1990a).

Shiga toxin was reported to cause damage to the central nervous system (CNS) in protein-deprived, malnourished mice (Kurioka *et al.*, 1998). In these mice that do not develop an effective intestinal barrier, oral infection with *E. coli* O157:H7 was shown to

result in neurological symptoms, detectable levels of toxin in the blood stream, and death with histological evidence of cerebral hemorrhage (Kurioka *et al.*, 1998). The authors of this report speculated that binding of Shiga toxin to cells of the vascular endothelium in the brain triggers apoptosis in cells that have been sensitized by circulating tumor necrosis factor alpha generated in response to the gastrointestinal infection. The result is loss of vascular integrity in the brain, hemorrhage, and death. Mice with proper nutrition that were fed *E. coli* O157:H7 did not succumb to infection or manifest any CNS symptoms (Kita *et al.*, 2000).

In a separate study, Shiga toxin-associated neuronal pathology was observed in mice fed *E. coli* O157:NM strain E32511/HSC that expresses Stx2c. Treatment with mitomycin C to enhance toxin expression resulted in damage to both neuron fibers and endothelium in the brain; this damage was not seen in infected control mice that were not treated with mitomycin C. Shiga toxin was localized to the damaged tissue as identified by mouse studies provide evidence that conditions that favor Shiga toxin absorption or expression increase the likelihood of central nervous system pathology in mice.

2. **90 kb plasmid**. The large plasmid of the EDL933 strain of *E. coli* O157:H7 has been sequenced and encodes 100 open reading frames, 19 of which have been described as potential virulence genes (Burland *et al.*, 1998). One of the potential plasmid-encoded virulence factors is the hemolysin operon (Schmidt *et al.*, 1995) that is well conserved among plasmids of many O157:H7 and non-O157 strains (Karch *et al*., 1998). Ninety-five percent of O157:H7 serotype plasmids also encode a putative type II secretion system, *etpC-P,* homologous to the pullulanase operon in *Klebsiella* (Schmidt *et al.*, 1997a). The frequency of the *etp* operon in non-O157 plasmids is 50%. The large

plasmids may also encode a catalase-peroxidase gene (Brunder *et al.*, 1996) and a serine protease gene (Brunder *et al.*, 1997), but these genes are detected in only 66% of the O157:H7 strains tested, and 38% and 36% respectively, of the non-O157 strains analyzed, an observation that indicates there is considerable heterogeneity among the plasmids of different STEC strains (Karch *et al.*, 1998). The role of hemolysin and these enzymes in pathogenicity has not been established. In addition to these defined genes, there is a large open reading frame (ORF) that encodes a region homologous to the active site of the large *Clostridium difficile* toxins A and B, however, a toxin has yet to be characterized.

Since the large plasmid of EPEC contains genes that influence adherence and A/E lesion formation of EPEC, many investigators have looked for adherence factors on the O157 plasmid with conflicting results. Karch *et al*. (1987) observed that a plasmid-cured O157:H7 strain had reduced adherence to Henle 407 cells and lost the ability to produce fimbriae, compared to the wild-type strain. In a different O157:H7 isolate, Toth *et al.*(1990) also observed reduced adherence in the plasmid-cured strain, but no fimbriae were seen in the wild-type strain. Tzipori *et al.*(1987) compared plasmid-cured and wild-type O157:H7 *in vivo* in gnotobiotic pigs to determine the influence of the plasmid on A/E lesion formation and diarrhea production and saw no differences. Wadlokowski *et al.* (1990) tested plasmid-cured and wild-type strains in the streptomycin-treated mouse model for STEC infection and saw a competitive advantage in colonization for wild-type when both strains were fed together. When the strains were fed individually, each strain colonized well.

3. Intimin. EHEC strains produce A/E lesions in the colonic epithelial tissue of infant animals including rabbits and piglets (Potter *et al.*, 1985; Francis *et al.*, 1986). These lesions are characterized by the loss of microvilli from the enterocyte brush border, actin reorganization within the enterocytes that results in pedestal formation around the infecting bacteria (Tzipori *et al.*, 1986). Jerse *et al.* identified intimin in EPEC, as the protein responsible for the intimate adherence of bacteria to the epithelial cells that is necessary to instigate actin rearrangement and pedestal formation characteristic of the A/E lesion (Jerse *et al.*, 1990; Jerse and Kaper, 1991). The intimin of EHEC is 88% similar overall to EPEC intimin and mediates the same type of A/E lesion formation and has also been identified as an important colonization factor for EHEC in piglets (McKee and O'Brien, 1995; McKee *et al.*, 1995).

4. The locus of enterocyte effacement (LEE). Intimin is necessary, but not solely responsible, for the formation of attachment and effacement lesions. It is one of a group of factors that mediate the formation of A/E lesions that are encoded within a 43 kb pathogenicity island known as the LEE. The LEE was first described in EPEC (McDaniel *et al.*, 1995). The EHEC LEE is analogous in organization and function to the EPEC LEE, but larger, due to 7.5 kb of prophage DNA on the 3'end. The LEE is comprised of three groups of genes. In the middle of the LEE are the intimin gene, *eae*, and *tir*, the translocated intimin receptor gene. Downstream of *eae* and *tir* are *espA*, *espD*, and *espB*. These Esp proteins are necessary for the signal transduction that results in A/E pathology in eukaryotic cells. Upstream of *eae* and *tir* are the *esc* and *sep* genes that encode a type III secretion system necessary for delivery of the *esp* gene products to the bacterial cell/host cell interface.

II. Shiga toxins

A. Structure and Function. Shiga toxins are AB_5 bipartite toxins in which the A subunit contains the enzymatic active site of the toxin and the B subunit is responsible for receptor binding. The A subunit is nicked by the action of cellular furin (Garred *et al.*, 1995) to yield A_1 and A_2 subunits that remain attached to one another by a disulfide linkage. The A_2 subunit interacts with the B pentamer. The A_1 subunit acts as an N-glycosidase and cleaves an adenine residue from the 28S ribosomal RNA of the 60S eukaryotic ribosomal subunit (Endo *et al.*, 1988; Endo *et al.*, 1987). As a result of this deadenylation, elongation factor-1-mediated binding of amino acyl-tRNAs cannot occur and protein synthesis ceases (O'Brien and Holmes, 1987). The modes of action of all Shiga toxins are the same, but the receptors they target differ slightly. The majority of Stxs bind the globotriaosylceramide receptor Gb_3 (Lindberg *et al.*, 1987; Waddell *et al.*, 1988), but the Stx2e variant (see below) binds globotetrasylceramide (Gb_4) (De Grandis *et al.*, 1989; Samuel *et al.*, 1990). Once the toxin is bound to its receptor, it is taken into the host cell by clathrin-mediated endocytosis and reaches the Golgi apparatus via the endosome (Sandvig *et al.*, 1993).

B. Classification and characteristics of Shiga toxins. Stx1 is virtually identical to Stx in that it differs by one amino acid in the A subunit. Although Stx1 and Stx2 share the same structure and activity, they are antigenically distinct. The nucleic acid sequences of *stx*$_1$ and *stx*$_2$ are approximately 56% identical to one another (Jackson *et al.*, 1987). Both Stx1 and Stx2 intoxicate Vero and HeLa cells and bind the same cellular receptor. Stx2 is thought to be more toxic than Stx1 based on epidemiologic data that show a

greater frequency of sequelae associated with Stx2-producing strains. In mice, Stx2-producers are more pathogenic that Stx1-producing strains (Boerlin *et al.*, 1999; Wadolkowski *et al.*, 1990a)

Stx1 is invariant, that is, those Stx1s made by diverse STEC isolates are essentially the same toxin. In contrast, several related variants of Stx2 have been described. These Stx2 variants include Stx2c, Stx2d, Stx2e and Stx2f (Schmitt *et al.*, 1991; Ito *et al.*, 1990; Melton-Celsa and O'Brien, 1996; Marques *et al.*, 1987; Schmidt *et al.*, 2000). The variant toxins are highly similar to Stx2 and are cross-neutralizable with anti-Stx2 polyclonal antibody, but they differ in biological activity [e.g. preferred cellular receptor, relative cytotoxicity for Vero and HeLa cells, and capacity to be neutralized by some anti-Stx2 monoclonal antibodies (Perera *et al.*, 1988)] or host range of strains that produce them. For example, Stx2c and Stx2d are made by STECs isolated from both humans and animals (Schmitt *et al.*, 1991; Pierard *et al.*, 1998). Stx2e is primarily made by STEC responsible for edema disease of swine, although infection with *E. coli* that produce the Stx2e variant is sporadically seen in humans as well. Stx2f is made by STEC strains isolated from feral pigeons but has also been associated with diarrhea in a child (Gannon and Gyles, 1990; Pierard *et al.*, 1998).

There are striking differences in the lethality for mice of STEC isolates that produce Stx2 variants. Indeed, STEC that make Stx2d are lethal for orally-challenged streptomycin-treated CD-1 mice at very low doses (Lindgren, 1993; Lindgren *et al.*, 1994), but STEC that synthesize Stx2 or the variants Stx2c or Stx2e, (Stx2f not tested) are only occasionally virulent at doses of about 10^{10} CFU/(CD-1 mouse). The lower mouse 50% lethal dose (LD_{50}) of STEC that produce Stx2d correlates with the capacity

of Stx2d to be activated by elastase derived from murine intestinal mucus (Melton-Celsa and O'Brien, 1996; Kokai-Kun *et al.*, 2000). Activation of Stx2d by elastase, which cleaves two amino acids from the C terminus of the Stx2d A_2 peptide, results in increased cytotoxicity of Stx2d to Vero cells (Melton-Celsa *et al.*, 2002).

C. Genetic organization of Shiga toxins. All Shiga toxin operons consist of *stxA* and *stxB* genes and share the same genetic organization. Putative promoters and transcriptional start sites have been mapped upstream of the *stx*, stx_1, stx_2, and stx_{2c} operons (Calderwood *et al.*, 1987; Sung *et al.*, 1990; Schmitt *et al.*, 1991). The promoters of *stx* and stx_1 contain an iron/co-repressor binding region (Calderwood and Mekalanos, 1987) not found in stx_2 or its variants. For each type of Shiga toxin, the toxin A subunit is encoded within an open reading frame approximately 960 bp in length that is separated by a gap of 12 –14 bp from the start of the 267 bp B subunit gene. The B subunit gene does not have its own promoter and is transcribed in a bicistronic message with *stxA*. Within the intercistronic gap, there is a ribosomal binding sequence (RBS) for translation of *stxB* that is stronger than the RBS responsible for translation of the A subunit mRNA. This arrangement provides for the differential translation necessary for assembly of the AB_5 holotoxin (Habib and Jackson, 1993).

D. Regulation of Shiga toxin expression.

1. Stx/Stx1 regulation. Expression of Stx and Stx1 is repressed by iron through the action of an iron-binding co-repressor protein, Fur, that acts on the specific iron regulatory *fur* sequence within the promoters of each toxin gene (Calderwood and Mekalanos, 1987; De Grandis *et al.*, 1987; Weinstein *et al.*, 1988). In addition to iron

repression, Stx production is affected by temperature, with significantly more toxin present in cultures grown at 37^{o} C compared to those grown at 30^{o}C. Temperature does not affect Stx1 production (Weinstein *et al.*, 1988). Bacteriophage induction exerts the most profound effect on Stx1 expression as is described below.

2. Stx2/Stx2 variant regulation. The expression of Stx2 is not influenced by iron concentration (Sung *et al.*, 1990). The regions upstream of Stx2 and the Stx2 variants 2c, 2d, and 2e differ significantly from the upstream regions of Stx and Stx1 and do not contain *fur* boxes (Jackson *et al.*, 1987; Schmitt *et al.*, 1991; Ito *et al.*, 1990; Muniesa *et al.*, 2000). For bacterial strains that harbor Stx2 or variant toxins within bacteriophages, phage induction wields the greatest influence on toxigenicity (see below) (Muniesa *et al.*, 2000; Muhldorfer *et al.*, 1996; Wagner *et al.*, 1999). The mechanism of regulation of Stx2 variant genes not located within bacteriophages is not known and represents a major area of investigation in this dissertation.

3. Bacteriophage regulation of toxin expression. Stx1 and Stx2 are encoded within two similar, but morphologically distinct, lambdoid bacteriophages (O'Brien *et al.*, 1984; O'Brien *et al.*, 1989). The toxin genes are located within the bacteriophage late gene cluster (Neely and Friedman, 1998; Plunkett III *et al.*, 1999). Because of this genetic arrangement, toxin gene expression is strongly influenced by the phage lytic cycle (Neely and Friedman, 1998; Wagner *et al.*, 2001). During lysogeny, the phage late gene cluster is repressed by the action of the phage-encoded repressor CI. CI binds the promoter, p_R', an event that prevents expression of the genes necessary for the phage lytic cycle. Circumstances that trigger the host cell SOS response upregulate

transcription of *recA*. The RecA protease cleaves CI, which, in turn, leads to transcription of the gene for the antitermination factor Q. Q modifies RNA polymerase at the late gene promoter p_R', and transcription proceeds beyond the strong transcription termination site t_R'. The toxin genes are then transcribed along with the late phage genes downstream of t_R' (Yarnell and Roberts, 1992; Fuchs *et al.*, 1999). In addition, the toxin genes are amplified through bacteriophage genome replication, and host cell lysis promotes toxin release (Neely and Friedman, 1998; Wagner *et al.*, 2001).

For a number of years, our laboratory and others thought that the variants of Stx2 produced by non-O157:H7 STEC were chromosomally encoded as is Shiga toxin in *Shigella dysenteriae* type 1. Recently, an inducible bacteriophage, ϕ27, bearing stx_{2e} from an STEC strain of human origin was isolated (Muniesa *et al.*, 2000). Expression of stx_{2e} in that strain is enhanced by bacteriophage induction, as expected, but no identifiable Q gene homologue is located upstream of stx_{2e}. Thus, the mechanism for regulation of late gene expression in ϕ27 remains unknown.

III. *Escherichia coli* strain B2F1.

A. Origin and virulence properties. This dissertation focuses on toxin production and regulation of toxin expression in an STEC O91:H21 strain B2F1. B2F1 was isolated from the stool of a child with HUS. The child and his family were believed to be infected through ingestion of moose meat (M.A. Karmali, personal communication). The strain, as mentioned earlier, belongs to the Whittam STEC 1 group and does not produce intimin but contains a large O157:H7-like plasmid that encodes hemolysin.

B2F1 carries two Stx2 variant toxin genes, stx_{2d1} and stx_{2d2}. These genes were originally sequenced by Ito, *et al.* who designated the two toxins VT2vha and –vhb, respectively (Ito *et al.*, 1990). The toxin genes were resequenced in our laboratory, and we found minor discrepancies in the sequences from those originally reported (Melton-Celsa *et al.*, 2002). DNA sequence comparison shows that stx_{2d1} and stx_{2d2} are 97% identical. The Stx2d1 and Stx2d2 proteins differ by one amino acid in the A subunit (position 18), a position that is not in the enzymatic active site as previously mapped for Stx2 (Jackson *et al.*, 1990). The B subunit genes are identical. The location of these two genes on the chromosome has not been determined. However, the toxin genes reside on different cosmids, an observation that suggests the genes are probably not adjacent to one another.

B. Stx2d activation. A model system to investigate the effects of STEC infection in mice was developed in this laboratory by Dr. Elizabeth Waldolkowki (Wadolkowski *et al.*, 1990b). Juvenile CD-1 mice are treated with streptomycin to reduce competition from normal intestinal flora and aid in the establishment of infection by oral challenge. In that model, Wadlolkowski *et al.* found that Stx2 produced by a hypervirulent O157:H7 isolate caused renal tubule necrosis (Wadolkowski *et al.*, 1990a). Subsequently, Lindgren *et al*. showed that strain B2F1 and another Stx2d-producing strain had an oral 50% lethal dose (LD_{50}) when fed to streptomycin-treated mice of less than 10 organisms. Conversely, STEC that produce Stx2 or Stx2c had an LD_{50} of 10^{10} organisms or greater (Lindgren *et al.*, 1993). In contrast, the Stx2d producers did not show elevated cytotoxicity *in vitro* compared to Stx2- or Stx2c-producers (Lindgren *et al.*, 1994). These observations led to further investigation of potential *in vivo* influences on toxin expression. Dr. Angela

Melton-Celsa showed that treatment of culture extracts from Stx2d-producing strains with intestinal mucus isolated from uninfected mice increased the cytotoxicity of that toxin 10- to 1000-fold (Melton-Celsa and O'Brien, 1996). The component of mucus responsible for activation was later identified as elastase (Kokai-Kun *et al.*, 2000). Recently, the site of elastase cleavage in the A_2 peptide of Stx2d was defined and activation was shown to be B pentamer-dependent (Melton-Celsa *et al.*, 2002). Also, human intestinal mucus activated the Stx2d toxins, as did human elastase, *in vitro* (Melton-Celsa and O'Brien, 1996; Kokai-Kun *et al.*, 2000). This latter finding raises the possibility that Stx2d toxins may be more pathogenic to humans than any other Shiga toxin due to elastase activation *in vivo* (Melton-Celsa and O'Brien, 1996; Kokai-Kun *et al.*, 2000).

C. Prior studies in B2F1 toxin regulation. Dr. Susanne Lindgren conducted some initial studies on regulation of Stx2d2 expression (included in her Ph.D dissertation in the laboratory of Dr. Alison D. O'Brien). She constructed a reporter plasmid that expresses an Stx2d2 translational fusion with alkaline phosphatase (pSQ448). She transformed pSQ448 into wild-type B2F1 and measured alkaline phosphatase expression under various growth conditions. She found that there was at most a two-fold increase in alkaline phosphatase produced at 37^{o}C versus 30^{o}C. This difference was not considered significant (Lindgren, 1993).

Dr. Lindgren also isolated stx_{2d2} from an stx_{2d2}-bearing cosmid by making a plasmid library of smaller fragments from that cosmid and screening transformants for cytotoxicity. Additional digests were done on these plasmids to isolate the smallest fragment of DNA that retained cytotoxicity. She discovered that the removal of a 1.9 kb

DNA fragment from directly upstream of *stx*$_{2d2}$ resulted in a toxin clone that was 100-fold more cytotoxic than the parent clone. She hypothesized that the upstream fragment encoded a repressor of toxin expression and cloned that region into a pKS$^-$ vector (pSQ19). Toxin expression was reduced by 25- to 41-fold when pSQ19 was co-transformed into strain DH5α along with an Stx2d2-expressing clone (pSQ547). The mechanism of this effect was not investigated, but these studies were the basis of further work that is described in this dissertation.

Dr. Lindgren's attempts to induce and isolate toxin-converting phage were not successful (Lindgren, 1993). She identified some homologous sequences to bacteriophage lambda in the B2F1genome but was unable to show any association of lambdoid DNA with *stx*$_{2d1}$ or *stx*$_{2d2}$. One part of this dissertation details my efforts, under the guidance of Dr. Clare Schmitt, to repeat the phage-induction studies and assess the influence of phage-induction on Stx2d expression.

IV. Specific aims of this dissertation

The major objective of my studies was to determine the mechanism of regulation of Stx2d toxin expression in *Escherichia coli* strain B2F1. We speculated that expression of the activatable Stx2d variant toxins might differ significantly from expression of the bacteriophage-borne Shiga toxins, Stx1 and Stx2. We hypothesized that Stx2d expression is regulated by a host cell-defined mechanism other than iron or temperature regulation. These theories were based on several preliminary observations in this laboratory. First, bacteriophage were not induced from B2F1 in initial studies (Lindgren, 1993). Second, mutation of the Stx2d2 allele ablated toxin production in B2F1 almost entirely even

though there was still an intact Stx2d1 allele, a finding that suggested down-regulation of the stx_{2d1} allele (A. Melton-Celsa, unpublished). Third, previously described environmental factors that affect Stx2 expression do not influence cytotoxicity of B2F1 (Lindgren, 1993). Fourth, Stx2d2 expression was repressed in the presence of a 1.9 kb region from upstream of stx_{2d2} (Lindgren, 1993).

To assess the basis of Stx2d1 and Stx2d2 regulation in *E. coli* strain B2F1, three specific aims were outlined. The first aim involved the construction of a knock-out mutation in stx_{2d1} of B2F1 (the stx_{2d2} mutation was constructed by Angela Melton-Celsa) so that expression of each toxin could be assessed independently *in vitro* and *in vivo* in mice.

The second aim was to test cytotoxicity of B2F1 and its mutants under conditions that induce bacteriophages and to reexamine the possibility that either toxin is bacteriophage-borne. Bacteriophage repression and induction exert the greatest influence on Stx2 expression, and we reasoned that bacteriophages, whether cryptic or inducible, could exert an influence on toxin expression in B2F1 or either of the mutants.

The third aim was to define the factor cloned from upstream of the Stx2d2 gene by Lindgren that reduced toxin expression when co-transformed with an Stx2d2 toxin gene clone *in trans* in a K-12 *Escherichia coli* (Lindgren, 1993). This third aim was modified and expanded as data were accrued. I made smaller clones from the 1.9 kb region upstream of stx_{2d2} associated with reduced Stx2d2 expression *in trans* in DH5α. One of the resultant clones, the one that contained the putative promoter of stx_{2d2}, was associated with reduced toxin expression when co-transformed into DH5α with the Stx2d2 toxin gene clone. From these data, I speculated that there is an activator of

Stx2d2 expression in K-12 that is competitively bound when additional copies of the promoter are provided *in trans*. This titration of an activator would result in a reduced level of expression from the toxin gene-associated promoter. Further, I postulated that B2F1 encodes an analogous activator that affects Stx2d2 expression in the wild-type background. Therefore, the third aim was extended to include identification of the putative activator gene of Stx2d2 expression in DH5α, mutation of the corresponding gene in B2F1, and assessment of the cytotoxicity and virulence *in vivo* in mice of the resultant B2F1 mutants.

MATERIALS AND METHODS

I. General laboratory procedures

A. Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this dissertation are summarized in Table 1 and Table 2. *E. coli* strain DH5α (written for brevity in this dissertation as DH5α) served as the usual host for recombinant plasmids except for clones derived with the Invitrogen TA Cloning Kit (Carlsbad, CA). For that kit, the competent host provided was InvαF'. *E. coli* strain B2F1 (also written simply as B2F1) was kindly supplied by Dr. M.A. Karmali. Strains that were used for *in vivo* studies in mice were spontaneously derived streptomycin-resistant (Str^r) mutants of B2F1 and DH5α and their derivatives (Lindgren *et al.*, 1993).

Escherichia coli DH5α, C600 and 395-1 were used as indicator strains for bacteriophage plaque detection. Strain C600 lysogenized with bacteriophage 933W [C600(933W)] was used to test lysates of B2F1 for phages related to 933W as defined by phage immunity patterns. Strain S17-1 λ*pir* served as the conjugational donor in the transposon mutagenesis of DH5α.

B. Media, enzymes and biochemicals. Cultures were incubated in Luria Bertani (LB) broth with aeration or on LB agar at 37°C except when otherwise specified (Sambrook *et al.*, 1982). Antibiotics (United States Biochemicals, Cleveland, OH) were added as needed for selection of strains or maintenance of plasmids at the following concentrations: ampicillin 100 μg/ml; chloramphenicol 30μg/ml; kanamycin, 25 μg/ml; naladixic acid, 15 μg/ml; streptomycin, 50 μg/ml; tetracycline, 12.5 μg/ml. Forty μl of 40

Table 1. Bacterial strains used in this study

Bacterial strains	Relevant characteristics	Reference
B2F1 Str^r	human isolate *E. coli* O91:H21 STEC encodes stx_{2d1}, stx_{2d2} Str^r	(Lindgren, 1993)
DH5α	K-12 strain *recA*$^-$ *E. coli* strain	(Hanahan, 1983)
DH5α Str^r	Spontaneously derived Str^r mutant of DH5α	(Lindgren, 1993)
DH5α(ϕB2F1)	K-12 strain, *recA*$^-$, lysogenized with ϕB2F1	This study
395-1	K-12 *E. coli* strain	(Sansonetti *et al.*, 1983)
C600	K-12 *E. coli* strain	(Smith *et al.*, 1983)
InvαF'	RecA$^-$ competent cells supplied with TA Cloning kit	Invitrogen
S17-1λ*pir*	K-12 *E.coli* with chromosomal RP4 transfer factor, lysogenized with λpir	(Miller and Mekalanos, 1988)
C600(933W)	lysogen of Stx2-converting phage 933W (O157:H7 strain 933EDL)	(O'Brien *et al.*, 1984)
DH5α mutant 4	Tn*5*-Km^r insertion in "div" gene in DH5α	This study
DH5α mutant 31	Tn*5*-Km^r insertion in *caiD* gene in DH5α	This study
DH5α mutant 38	Tn5-Km^r insertion in *ycdU* gene in DH5α	This study
B2F1 mutant 1-1	stx_{2d1}::*cat* toxin knock-out, expresses Stx2d2	This study
B2F1 mutant 7-4	stx_{2d2}::*cat* toxin knock-out, expresses Stx2d1	This study
B2F1 mutant 4	Mini-Tn*5* Km^r insertion in div gene acquired by allelic exchange from homologous mutation in DH5α	This study
B2F1 mutant 31	Mini-Tn*5* Km^r in *caiD* gene acquired by allelic exchange from homologous mutation in DH5α	This study

Table 2. Plasmids used in this study

Plasmids	*Relevant characteristics*	*Reference*
pUC18	Am^r	(Norander *et al.*, 1983)
pCM4	Am^r, Cm^r, Tc^r	Pharmacia
pKS/SK	Bluescript vector, high copy number, Am^r, *Kpn*1-*Sal*1 or *Sal1*-*Kpn*1 orientation of multiple cloning site, respectively	Stratagene
pBC	Bluescript vector, high copy number, Ch^r	Stratagene
pACYC184	Medium copy number, $Ch^{r,}$ \ Tc^5	New England Biolabs
pMAK705	Km^r, *ori* $_{ts}$	(Hamilton *et al.*, 1989)
pJES210	stx_{2d2} cosmid clone in pHC79, Am^r	This study
pMJ100	stx_2 clone is Bluescribe BS, Am^r	(Weinstein *et al.*, 1989)
pMJSK	stx_2 clone from pMJ100 in pKS, Am^r *Hind*III, *Eco*RI in opposite orientation to vector *lacZ* promoter	This study
pSQ12	stx_{2d1} cosmid clone in pHC79, Am^r	(Lindgren *et al.*, 1993)
pSQ19	1.9 kb region from upstream of stx_{2d2} cloned into Bluescript at *Eco*RV and *Sal*I sites, Am^r	(Lindgren, 1993)
pSQ343	stx_{2d1} in Bluescript KS^-, Am^r on 2.3 kb *Pst*I-*Eco*RI fragment	(Lindgren *et al.*, 1993)
pSQ347	stx_{2d1} in pACYC184, Cm^r on 2.3 kb *Bam*HI-*Eco*RI fragment	(Lindgren, 1993)
pSQ544	stx_{2d2} in Bluescript SK^-, Am^r on 4.0 kb *Sal*I-*Eco*RI fragment	(Lindgren, 1993)
pSQ545	stx_{2d2} in Bluescript KS^-, Am^r on 2.8 kb *Pst*I-*Eco*RI fragment	(Lindgren, 1993)
pSQ547	stx_{2d2} in pACYC184, Cm^r on 2.8 kb *Bam*HI-*Eco*RI fragment	(Lindgren, 1993)

Table 2., continued

pMB100	stx_{2d2} in Bluescript with blunt-ended *cat* inserted into *Eco*RV site, Am^r	This study
pMB101	pUC18 with stx_{2d1},Am^r	This study
pMB102	stx_{2d1} in pUC18 with blunt-ended *cat* inserted into blunted *Ava*1 and *Acc*1 sites , Am^r	This study
pSTAMP	derived from pMAK705 Km^r was replaced with Am^r ori_{ts}	This study
pLT8	190 bp tRNA region from pSQ19 in Bluescript KS^-, Am^r	This study
pLTF	190 bp tRNA region from pSQ19 in Bluescript SK^-, Am^r	This study
pBP	306 bp putative promoter region from pSQ19 in Bluescript KS^-, Am^r	This study
pHP	306 bp putative promoter region from pSQ19 in Bluescript SK^-, Am^r	This study
pLT12	1.3 kb methyl transferase ORF from pSQ19 in Bluescript KS^-, Am^r	This study
pLT13	1.3 kb methyl transferase ORF from pSQ19 in Bluescript SK^-, Am^r	This study
pLT604	600 bp region of scrap vector DNA from pSQ19 in Bluescript, KS^-, Am^r	This study
pAM450	pSTAMP with *sacB/R* cloned in at *Pst*I site	(McKee *et al.*, 1995)
pMB103	pAM450 with mutated stx_{2d2} inserted into *Sal*I and *Bam*HI sites	This study
pLT10	pSTAMP with mutated stx_{2d1} inserted into *Kpn*I and *Pst*I sites	This study
pIM10	*Escherichia coli recA* clone	(Fuchs *et al.*, 1999)
pQF50	Reporter vector backbone *trp* terminator sequences, multiple cloning site and *lacZ*	(Farinha and Kropinski, 1990)
pATM161	pUT vector with RP4 *mob*, R6K *ori*, mini-Tn*5* Km^r, Am^r	(De Lorenzo *et al.*, 1990)

Table 2., continued

pLTRC	Reporter plasmid derived from pQF50 with stx_{2d2} promoter::*lacZ* in pBC vector backbone	This study
pRV4	pKS$^-$ with DH5α Tn5-Km^r mutation 4 in *Eco*RV	This study
pRV31	pKS$^-$ with DH5α Tn5-Km^r mutation 31 in *Eco*RV	This study
pRV38	pKS$^-$ with DH5α Tn5-Km^r mutation 38 in *Eco*RV	This study
pLT14	pSTAMP with DH5α Tn5-Km^r mutation 4 on *Eco*RV fragment at *Hinc*II	This study
pPLT31	pSTAMP with DH5α Tn5-Km^r mutation 31 on *Eco*RV fragment at *Hinc*II	This study
pLT38	pSTAMP with DH5α Tn5-Km^r mutation 38 on *Eco*RV fragment at *Hinc*II	This study
pWKS130	Km^r low copy number vector	(Wang and Kushner, 1991)
pYCDU	*ycdU* gene from DH5α in pWKS130 Cm^r	This study
pCAID	*caiD* gene from DH5α in pWKS130 Cm^r	This study
pCAIE	*caiE* gene from DH5α in pWKS130 Cm^r	This study
pCAIDE	*caiD* and *caiE* genes from DH5α in pWKS130 Cm^r	This study

μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Sigma, St. Louis, MO) was spread onto the surface of prepared agar plates to distinguish transformed colonies that contained Bluescript (Stratagene, La Jolla, CA) or pCR2.1 (Invitrogen) cloning vectors without insertions in *lacZ* (blue colonies) from transformants in which DNA had successfully been inserted into the vector *lacZ* gene (white colonies). X-gal was also added to LB agar as described to screen for beta-galactosidase production with an stx_{2d2} promoter::*lacZ* transcriptional fusion reporter plasmid (see below). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4 ligase was purchased from United States Biochemicals. Calf intestinal alkaline phosphatase, RNase, and glycogen were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

C. Recombinant DNA techniques. Plasmid DNA was isolated by alkaline-lysis (Sambrook *et al.*, 1982) or by the Qiagen Miniprep procedure according to manufacturer's instructions (Qiagen, Valencia, CA). Chromosomal DNA was isolated by the small-scale procedure for bacterial chromosomes (Ausubel *et al.*, 1989). Briefly, broth-grown bacteria were harvested by centrifugation and disrupted at 65^{o}C in Tris/EDTA buffer (10mM Tris·Cl, pH 7.5, 1mM EDTA, pH 8.0) that contained sodium dodecyl sulfate (SDS) and Proteinase K. The nucleic acids were selectively precipitated from this lysate by the addition of 5M NaCl and 10% cetyltrimethylammonium bromide (CTAB), and then extracted with phenol/chloroform and precipitated in isopropanol. DNA fragments were separated by agarose gel electrophoresis in the presence of ethidium bromide so that fragments could be visualized in UV light and then excised according to size and eluted from the gel on GeneClean Spin columns (Bio 101, Carlsbad, CA). Ligation reactions were done at 14^{o}C and DNA from the cloning vector

was treated with alkaline phosphatase after single restriction enzyme digestions to prevent self-religation.

Polymerase chain reaction (PCR) amplification was used to screen transformants or transductants for acquisition of particular target genes as well as to generate DNA probes and sequences for cloning purposes. The primers used for PCR amplification are listed in Table 3. AmpliTaq and GeneAmp reagents by Perkin Elmer (Roche, Branchburg, NJ) were used for PCR reactions done in either a Perkin Elmer thermocycler (Norwalk, CT) or Minicycler by MJ Research, Inc. (Watertown, MA). The Invitrogen TA Cloning Kit was used to create clones of PCR-amplified products derived from pSQ19 and to clone PCR-derived genes from DH5α and C600(933W). The pCR2.1 vector is provided as a linear molecule with overhanging 3' thymidine residues at each end into which *Taq* polymerase-generated products that contain an extra 5' adenosine are readily ligated. Each PCR-derived insertion in pCR2.1 was sequenced to check for errors and the error-free PCR-derived insertion was released from pCR2.1 by digestion with restriction enzymes and ligated into Bluescript KS^- that had been similarly digested for additional studies. In the event that PCR-derived clones had incorporated minor errors in sequence during amplification, sequences were corrected by site-directed mutagenesis with the Strategene Quick Change kit according to the manufacturer's instructions.

DH5α was made competent for transformation by calcium chloride treatment and heat shock as described (Mandel and Higa, 1970). B2F1 is not readily transformed by calcium chloride treatment and heat shock and, therefore, was made competent for electroporation as described Chuang, *et al.* with the following modifications (Chuang *et al.*, 1995). Bacteria were grown in LB at 30^oC, subjected to heat shock at 37^oC, and then

Table 3. PCR primers used in this study

Primer pairs and sequences	Amplification products	Reference for sequence
CKS1-TGAGAGCGATCGACTCATAAT CKS2-GACTGAATTGTGACACAGATTA	entire *stx*$_{2d}$ genes	This study
LT2-CAGATAATCAGTGCGAGC JCS2- ACTCCGGAAGCACATTGC	a 1.2 kb internal stx$_{2d}$ fragment	This study
LTA-CATCCGTTCTGACTGGC LT7-TGCATTAGGAAGCAGC	methyl transferase ORF from pSQ19	This study
LTF-GAACAGCTGGCGCTCTGTC LT4-TCAGGGTTTAGCCAGTC	tRNA region from pSQ19	This study
LT8-CAGAGCGCCAGCTGTTCGA PE II1-ACCCAGTAACAGGCACAGTACC	Putative *stx*$_{2d2}$ promoter from pSQ19	PEII-1(Sung *et al.*, 1990)
LTD- ACATGGATCCTCTACGC T7 –GTAATACGACTCACTATAGGGC	600 bp fragment of vector origin on pSQ19	This study
Q up-ACGGTGTTCTTATGGTTCACC Q dn-CCACTCTTATCATGATATGC	Q gene from 933W	(Plunkett III *et al.*, 1999)
KUP-ATGATTGAACAAGATGGATTGC KDN-TCAGAAGAACTCGTCAAGAAGGC	neomycin/kanamycin phosphotransferase from the mini-Tn*5* Km^r	(De Lorenzo *et al.*, 1990)
RT5U-TCGCAACATCCGCATTAACAATC TN5D-GATGTTACCGAGAGCTTGGTAC	outward from mini-Tn*5* Km^r transposon ends	(De Lorenzo *et al.*, 1990)
YCD5-ATACATTTCTGAATATGCCAAC YCD3-TGCGCAAGAGAGTACAGAAC	*ycdU* gene from DH5α and B2F1	(Blattner *et al.*, 1997)
LT54-CAAGACGGCACTGGGATGGGAC LT55-TGGAGATCAGAAAAGATCTGC	*caiD* gene from DH5α and B2F1	(Blattner *et al.*, 1997)
1142-CCAACGGCTAAATAAGTC 2332-GCTGGTATTATTGTCACG	"div" gene from DH5α and B2F1	(Blattner *et al.*, 1997)

the heat-shocked organisms were harvested by centrifugation and resuspended in 10% glycerol prior to freezing at -80^{o} C for subsequent transformation with various recombinant or vector-control plasmids. Transformation of B2F1 was accomplished by electroporation with a Bio-Rad Gene Pulser (Hercules, CA) under the following conditions: 25μFD, 1.25 KV, 1000 Ohms.

D. DNA Hybridization. Southern analyses were used to verify that mutational insertions of the appropriate size had been made within the individual toxin genes of B2F1 following mutation by allelic exchange, to identify the sites of Tn*5* insertional mutations in DH5α, and to demonstrate that transposon mutations from DH5α had successfully been moved into the chromosome of B2F1. Chromosomal DNA was isolated from broth-grown organisms and the DNA was then digested to completion with restriction enzymes. The resulting fragments were separated by electrophoresis in 0.8% agarose gels and transferred by capillary action to nitrocellulose with the Turboblotter System (Schleicher and Schuell, Keene, New Hampshire). B2F1 toxin mutants were probed with the stx_{2d1} gene and chloramphenicol acetyl transferase (*cat*) gene, and transposon mutants of DH5α were probed with the kanamycin resistance gene. To verify the insertion of the DH5α mutations into the B2F1 chromosome, Southern blots were probed with the *caiD* or "div" genes that were PCR-amplified from DH5α chromosomal DNA.

Dot blot hybridization was used to assess differences in toxin gene copy number between B2F1 and the toxin mutants with and without mitomycin C induction. Bacteria grown in broth cultures were disrupted by sonication. The lysates were then clarified by centrifugation, serially diluted, and applied to nitrocellulose membranes with a vacuum

manifold. The membranes were dried and DNA was denatured with 0.5 M NaOH, then the blots were probed with an stx_{2d1} DNA probe or a *cat* DNA probe and the intensity of the signal was compared visually to known concentrations of control plasmid DNA to assess increased gene copy with induction.

Colony hybridization was used to detect DH5α colonies transduced with the stx_{2d1}-bearing phage. Colonies of putative lysogens were lifted from agar plates onto nitrocellulose filters. The filters were treated with 0.5 M NaOH to lyse the colonies and denature the DNA. The membranes were then washed in 5X salt sodium citrate solution (0.75 M sodium chloride, 75 mM sodium citrate, pH 7.0) and probed with the stx_{2d1} toxin gene.

The $stx_{2d,}$ kanamycin, *caiD* and "div" gene probes used for Southern and dot blots were generated from PCR-derived DNA products (Table 3). The *cat* gene probe was digested from pCM4 with *Bam*HI. In each case, the probes were labeled with the Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labeling System reagents (Amersham Life Science, Buckinghamshire, England). DNA-DNA hybridization reactions were detected with the ECL Detection System according to the manufacturer's instructions (Amersham Life Science, Buckinghamshire, England).

E. DNA Sequencing. First, the nucleic acid sequence of the 1.9 kb fragment from upstream of stx_{2d2} in pSQ19 (Lindgren, 1993) was determined. Later in this project the regions 4 kb upstream and downstream of each of the stx_{2d} genes were sequenced as well. The cosmid clones bearing either toxin (pSQ12 and pJES210) served as templates for the sequencing reactions. The upstream and downstream sequences flanking stx_{2d1} and

stx$_{2d2}$ were submitted to the GenBank under accession numbers AF479828 and AF479829, respectively. DNA sequencing was also used to verify that PCR-derived genes to be cloned were free of errors (see previous section) and to identify genes flanking transposon insertions in DH5α. In each case, sequencing was done with the ABI Prism or Big Dye Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) with primers synthesized on the ABI Nucleic Acid Synthesizer Model 394 or 3948. The products were separated and analyzed with the Applied Biosystems Model 377 or 3100, and the sequence results were aligned and compared with those in GenBank of the National Center for Biotechnology and Informatics (NCBI) by the BLAST program version 2.2.1 (Altschul *et al.*, 1990) or the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, WI).

F. Beta-galactosidase assay. Beta-galactosidase assays were done as outlined by Miller to determine the level of beta-galactosidase produced by pLTRC in the native DH5α background compared to the level of beta-galactosidase expressed in the DH5α mutants (Miller, 1972). Briefly, optical densities of LB broth cultures incubated overnight were measured at 600 nm, then 0.1 or 0.5 ml of broth culture was diluted to 1.0 ml in Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM NaH_2P0_4, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol). These samples were then treated with 2 drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate (SDS) to lyse cells. After the lysed cells were allowed to equilibrate at 30^o C for 5 minutes, 0.2 ml of a 4 mg/ml solution of the beta-galactosidase substrate ortho-nitrophenol-β-D-galactoside (ONPG, Sigma) was added. The reaction was timed and allowed to proceed at 30^{o}C until a yellow color was apparent. The reaction was stopped with 1.0 M Na_2CO_3, and absorbance was

measured at 420 nm and 550 nm for each sample. The Miller units were determined according to the formula $1000 \times (OD_{420} - 1.75 \times OD_{550}) / time_{sec} \times inoculum\ vol_{ml} \times OD_{600}$.

II. Assessment of toxicity of STEC *in vitro* and *in vivo*

A. Cytotoxicity measured by Vero cell assay. Bacterial cell lysates were prepared by sonically disrupting cells in broth cultures. The lysates were then centrifuged to remove cellular debris. To determine toxin levels in mouse feces, pellets were collected, weighed and suspended in sterile saline to make a 1:10 dilution (w/v). The suspensions were homogenized with a vortex mixer, and the supernatants were sterilized by passage through a 0.45 μm filter. The supernatants from cultures or fecal extracts were serially diluted in tissue culture medium and inoculated into wells of microtiter plates that had been seeded with 10^4 Vero cells per well 24 hours prior to addition of the toxin-containing materials (Gentry and Dalrymple, 1980). After 48 hours of incubation the remaining cells in each well were fixed in 10% formalin, stained with crystal violet, and the absorbance at 600nm was measured in each well with an automated ELx800 microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT). The reciprocal of the dilution that caused death of 50% of the cells in the monolayer compared with control wells was expressed as the 50% cytotoxic dose (CD_{50}) per milliliter of culture lysate or CD_{50} per ml fecal extract from a gram of feces. Thus, an increase in CD_{50}/ ml represents an increase in toxicity and lower values of CD_{50} represent less toxicity. Assays were done at least three times and the geometric means were calculated from the log values of CD_{50}/ml lysate or fecal extract. The 95% confidence intervals were determined from the

standard errors of the geometric mean of each group. In mice treated with subinhibitory concentrations of ciprofloxacin, the ratio of the logs of CD_{50} to CFU was calculated per gram of fecal pellets.

B. Mouse model of STEC infection. The streptomycin-treated mouse model of STEC infection (Wadolkowski *et al.*, 1990b) was used to assess virulence of B2F1, the individual toxin-producing mutants, and the transposon mutations from DH5α that had been moved into B2F1 by allelic exchange. Briefly, juvenile CD-1 male mice were fed streptomycin water (5 g/L) and food was withheld overnight to reduce normal gut flora. The following day streptomycin- resistant bacterial strains that had been grown overnight in LB broth were diluted to the desired concentration in saline and then suspended in a 20% sucrose solution that was fed to the mice. The mice were then permitted food *ad libitum* but maintained on streptomycin water for the duration of the experiment.

To compare the 50% lethal dose of orally administered B2F1 with that of the Stx2d1-producing mutant (7-4) and the Stx2d2-producing mutant (1-1), groups of five mice each were fed a range of doses from 10^2 to 10^8 CFU of each strain. A control group of five mice also received oral streptomycin but was not infected with bacteria. The mice were monitored for three weeks and mean times to death per group were calculated. The LD_{50} for each strain was calculated by the Reed and Meunsch method for determination of 50% endpoints (Reed and Muench, 1938). The second day after mice were challenged with bacteria, fecal pellets were cultured to establish that each group had become colonized. A similar experiment was conducted to compare the virulence in mice of wild-type B2F1 with that of the *caiD* and "div" gene mutants of B2F1. This experiment

differed from above in that the range of bacterial inocula fed was 10^3 to 10^8 CFU per mouse.

Transposon mutants of DH5α that showed reduced cytotoxicity *in vitro* compared to wild-type DH5α similarly transformed with the plasmid-borne stx_{2d2} gene (pSQ545) were fed to mice to determine whether virulence was also reduced in the DH5α mutants. Ampicillin was added to the drinking water at the concentration of 5g/L to provide selective pressure for the retention of toxin-encoding plasmids .

To assess the influence of subinhibitory doses of ciprofloxacin on virulence of the Stx2d1-producing B2F1 mutant (7-4), we used a modified version of the protocol described by Zhang, *et al.*(2000). These investigators tested the influence of ciprofloxacin therapy on *in vivo* Stx2 expression by *E. coli* O157:H7 (Zhang *et al.*, 2000). In this study, the subinhibitory dose of ciprofloxacin for Stx2d1-producing B2F1 mutant (7-4) was defined as that concentration that decreased fecal bacterial counts by a factor of 10 to 1000. The timing of the dosing of ciprofloxacin to achieve this reduction in CFU/g feces was determined in a pilot study and was different from that used by Zhang and colleagues. The requirement for such an adjustment in the dosing schedule probably reflects the fact that *in vitro E. coli* O157:H7, unlike B2F1 or the Stx2d1-producing B2F1 mutant (7-4), readily lyses after ciprofloxacin induction. For these mouse experiments, twenty animals were fed 10^7 organisms (day 0). Ten mice were then treated intraperitoneally (ip) on days 2,3,4, and 5 with 40 μg of ciprofloxacin (in 100 μl sterile water) while the other ten received 100 μl ip injections of sterile saline according to the same schedule. Five additional control mice received ciprofloxacin injections but no bacteria. The actual dose of bacteria given to each group of animals was calculated

retrospectively based on the CFU/ml of the original overnight broth culture. The mice were assessed daily for signs of illness and death over a three-week period, and fecal pellets were obtained on days two through five and again on day nine to quantitate CFU/g feces. Note that moribund animals typically stopped producing fecal pellets. The LD_{50} was calculated by the Reed and Meunch method for computation of 50% endpoints (Reed and Muench, 1938). Fecal cytotoxicity levels were assayed as detailed above.

III. Mutagenesis of *E. coli* strains B2F1 and DH5α

A. Construction of individual toxin-producing mutants of strain B2F1.

Individual mutants of B2F1 that produced either Stx2d1 or Stx2d2 were derived by allelic exchange as follows. Suicide vectors, pSTAMP and pAM450, were constructed by Angela Melton-Celsa as follows. The kanamycin resistance marker from the temperature sensitive vector pMAK705 (Hamilton *et al.*, 1989) was replaced with the beta-lactamase gene from pUC18. This step was necessary because B2F1 gives rise to kanamycin resistant derivatives at a high frequency (Melton-Celsa, unpublished observation). A sucrose sensitivity allele, *sacB/R*, was then inserted into the *Pst*I site in pSTAMP to give rise to pAM450, a suicide vector resembling those described by Blomfield, *et al.* (McKee *et al.*, 1995; Blomfield *et al.*, 1991). Although the original purpose of introducing the *sacB/R* allele into pSTAMP was to permit positive selection against transformants that retained the *sacB/R* allele (such as an unresolved co-integrate), we subsequently found that B2F1 was not sensitive to high concentrations of sucrose even in the presence of s*acB/R*. Nevertheless, both pSTAMP and pAM450 (without benefit of the sucrose selection) were used to facilitate allelic exchange in B2F1.

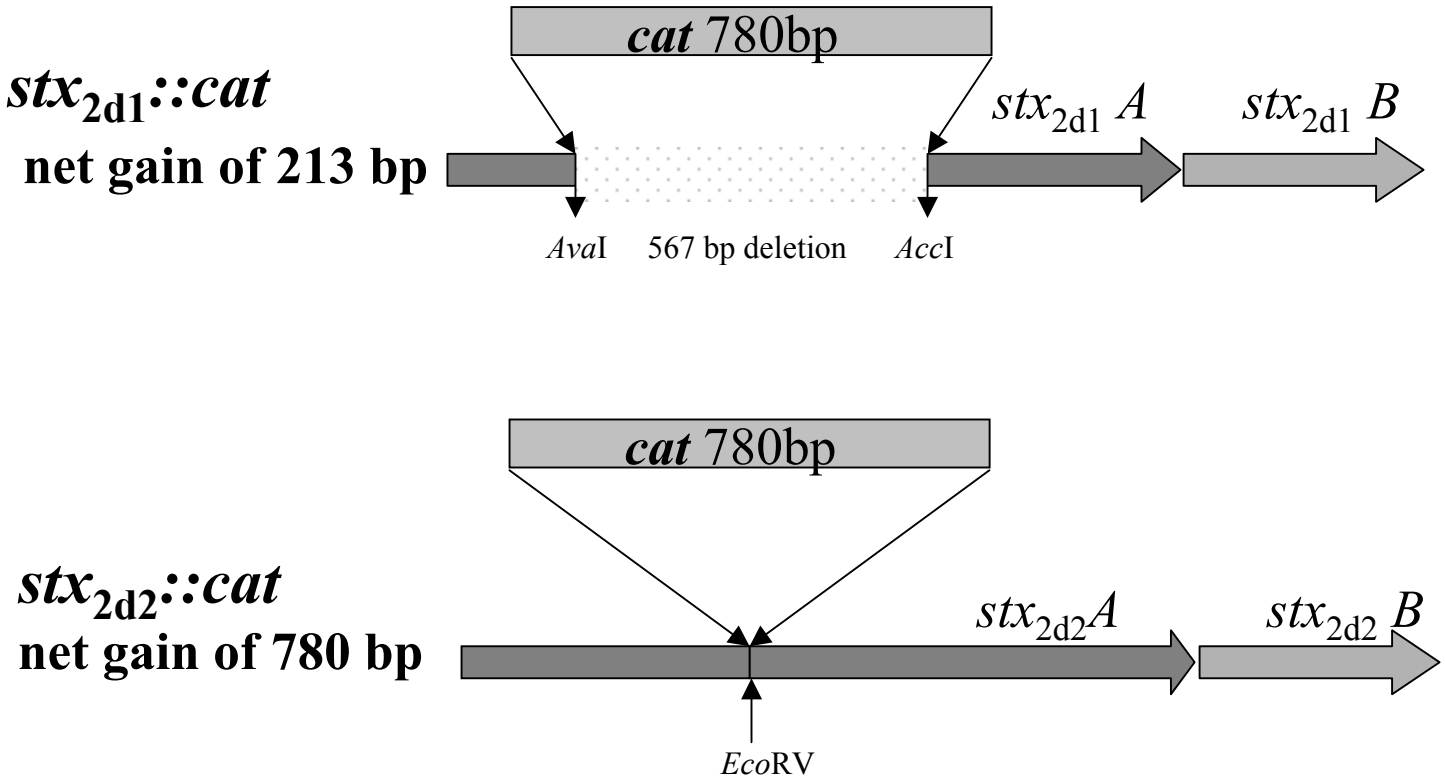


Figure 1. Diagram of the mutations introduced into *stx*$_{2d1}$ and *stx*$_{2d2}$. Mutations in the B2F1 toxin genes were made by insertion of the chloramphenicol acetyl transferase (*cat*) gene into the A subunit coding regions of *stx*$_{2d1}$ and *stx*$_{2d2}$.

The toxin genes were interrupted by the insertion of the chloramphenicol acetyl transferase cassette into the A subunit sequence of each toxin gene (Figure 1). The *cat* cassette was released from pCM4 (Phamacia) by digestion with *Bam*HI. The DNA polymerase I Klenow fragment (Boehringer Mannheim, Indianapolis, IN) was used to blunt the staggered ends that resulted from cleavage with *Bam*HI. The *cat* cassette was then ligated into pMB101 (stx_{2d1}) that had been digested with *Ava*I and *Acc*I, and treated with Klenow to yield compatible blunt ends (net gain of 213 bp) or into pSQ544 (carries stx_{2d2}) at the *Eco*RV site (a gain of 780 bp) to make pMB102 and pMB100, respectively. The mutated toxin genes were then subcloned into pSTAMP or pAM450, respectively.

B2F1 was transformed with the resulting clones (pLT10 or pMB103) by electroporation (as described above). Putative cointegrates were selected for vigorous growth during incubation at 44^{o}C in the presence of 100 μg/ml ampicillin. Co-integrates were then resolved by several rounds of growth at 30^{o}C in LB broth supplemented with chloramphenicol (15 μg/ml). Chromosomal DNA isolated from the putative mutants (sensitive to ampicillin with low-level resistance to chloramphenicol) was digested with *Pst*I and screened by Southern blot analysis. In wild-type B2F1 the stx_{2d1} gene resides on a 4.3 kb *Pst*I DNA fragment and the stx_{2d2} gene is located on a 4.9 kb fragment. With the mutagenic insertions, the expected fragment sizes for the stx_{2d1}-bearing fragment were 4.5 kb and 5.7 kb for the stx_{2d2} fragment (Fig. 2.).

B. Transposon mutagenesis of strain DH5α. We had observed that cytotoxicity of stx_{2d2} clones in DH5α decreased when DH5α was co-transformed with a plasmid bearing the 300 bp region from upstream of stx_{2d2}. We speculated that this reduction in cytotoxicity resulted from competition between the toxin gene clone and additional

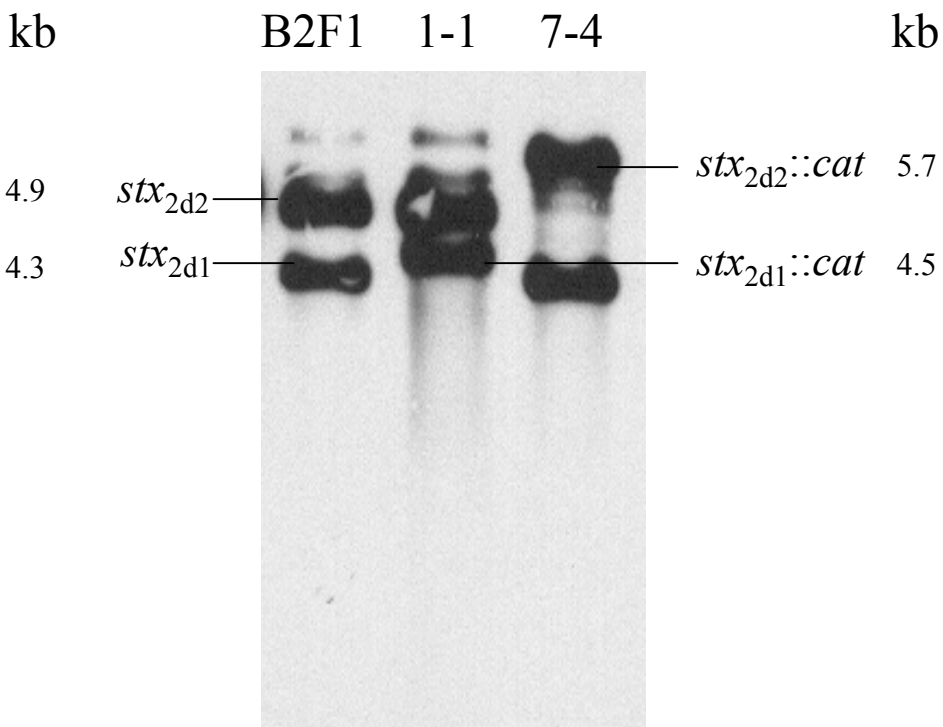


Figure 2. Southern blot showing the insertional mutations in the Stx2d genes ofstrain B2F1. Chromosomal DNA from wild-type B2F1, the *stx*$_{2d1}$::*cat* mutant (1-1), and the *stx*$_{2d2}$::*cat* mutant (7-4) was digested with *Pst*I and the blot was probed with a 1.2 kb PCR-amplified fragment of *stx*$_{2d1}$ labeled with the ECL Chemiluminescence enzymatic label (Amersham).

copies of the putative *stx*$_{2d2}$ promoter for a transcriptional activator encoded by DH5α. Therefore, I made random transposon mutations to inactivate genes in DH5α that might positively influence expression from the *stx*$_{2d2}$ promoter. I identified such potential mutants by screening for colonies that showed decreased *lacZ* expression from an *stx*$_{2d2}$ promoter and *lacZ* transcriptional fusion on a reporter plasmid (pLTRC) in the presence of X-gal.

1. Construction of an *stx*$_{2d2}$ reporter plasmid. A reporter plasmid was constructed to study factors that influence transcription from the *stx*$_{2d2}$ promoter region. First, an *stx*$_{2d2}$::*lacZ* fusion was constructed in pQF50 (Farinha and Kropinski, 1990), as shown in Figure 3A. This vector contains tandem synthetic *trp* termination sequences followed by a multiple cloning site located upstream of a promoterless *lacZ* gene in a pUC18-based vector backbone with an ampicillin resistance marker. The *trp* sequences prevent transcription that is initiated at the beta-lactamase (Am^r) gene promoter from proceeding through *lacZ*. A 667 bp *Sma*I fragment from pSQ544 (extending from 600 bp upstream of the Stx2d2 A subunit coding sequence to 67 bases downstream of the A subunit start sequence) was cloned into pQF50 at the *Sma*I site in the multiple cloning site region. This 667 bp *Sma*I fragment contained the putative *stx*$_{2d2}$ promoter based on the transcriptional start site mapped for *stx*$_2$ (Sung *et al.*, 1990); *stx*$_2$ is 96% homologous to *stx*$_{2d2}$ in that region. *E. coli* DH5α transformants with pQF50 that contained the *Sma*I-*stx*$_{2d2}$ fragment in the correct orientation produced blue colonies on LB agar with ampicillin in the presence of 40 μg/ml X-gal. The orientation of the insertion in pQF50 was verified by DNA sequencing, and the plasmid was renamed pQF50.1.

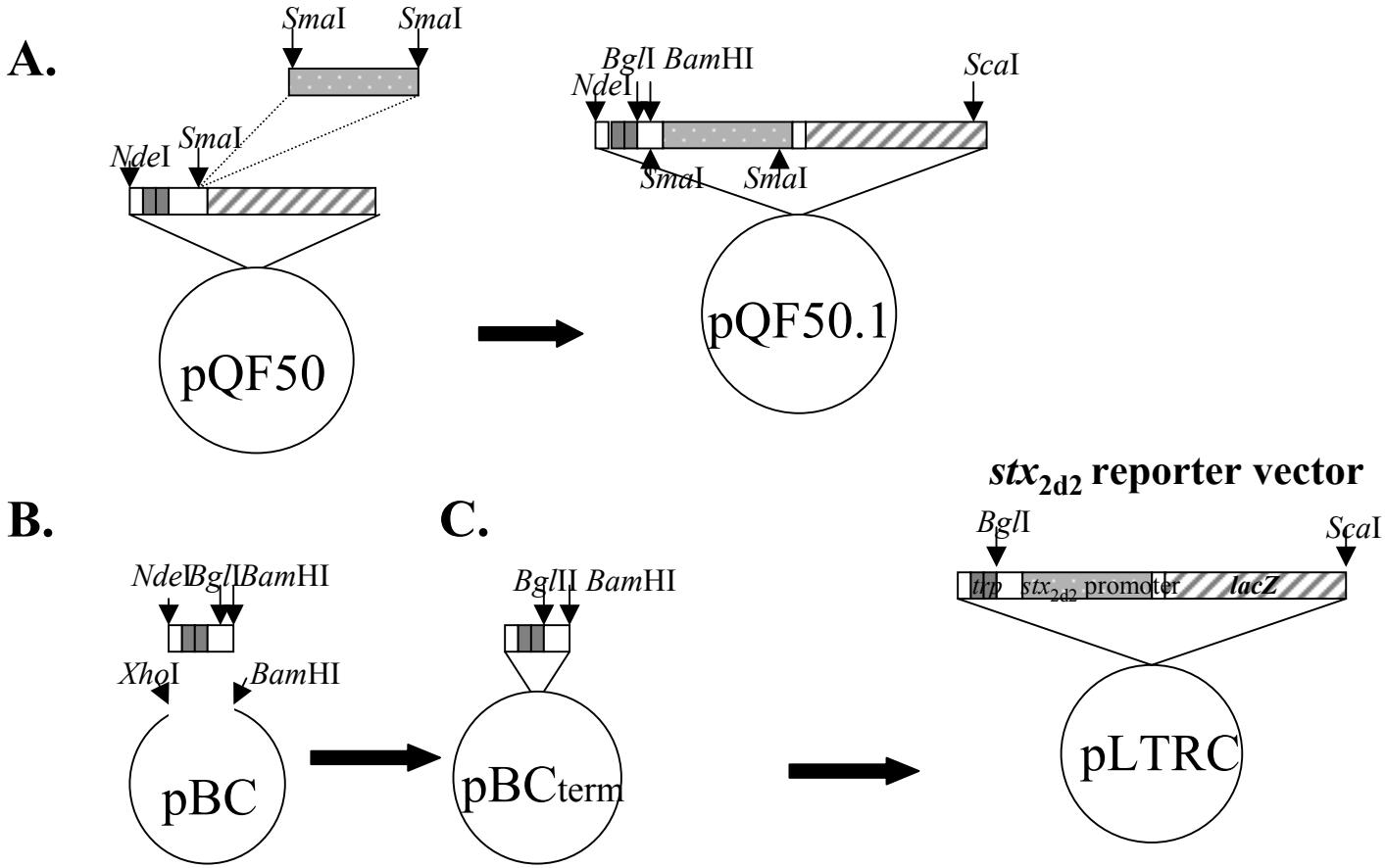


Figure 3. Construction of an *stx*$_{2d2}$ promoter reporter plasmid. **A.** Insertion of *stx*$_{2d2}$ promoter into pQF50 at *Sma*I site upstream of *lacZ* to make pQF50.1. **B.** Transfer of *trp* transcription terminators into pBC from pQF50.1 by ligation of Klenow- treated blunt ends (*Nde*I and *Xho*I) and *Bam*HI restricted ends to make pBCterm. **C.** Transfer of the s*tx*$_{2d2}$ promoter::*lacZ* to make pLTRC by ligation of *Bgl*II digested ends and ligation of the *Sca*I end of the reporter fragment to the Klenow-treated *Bam*HI end of pBCterm.

Next the reporter cassette (comprised of the transcription termination sequences and stx_{2d2} promoter::*lacZ* fusion) was moved into the Bluescript vector pBC that contains a chloramphenicol resistance gene. This change in antibiotic resistance genes was necessary because ampicillin selection could not be used in the transposon mutagenesis scheme summarized in Figure 4. The transfer of the reporter cassette from pQF50.1 to pBC was accomplished in two steps. First, the transcription terminators (100 bp fragment) were digested from pQF50.1 by sequential treatment with *Nde*I and *Bam*HI. The terminator fragment was then ligated into pBC that had been digested with *Xho*I and *Bam*HI (the *Nde*I- and *Xho*I-digested ends were treated with Klenow to create blunt-ended fragments compatible for ligation to one another) (Fig. 3B). I selected DH5α transformants on chloramphenicol agar that produced white colonies on X-gal, an indication that an insertion had interrupted the pBC *lacZ*. Plasmid DNA was purified from those transformants and digested with *Bgl*II to show that pBC had acquired the unique *Bgl*II site that adjoined the transcription termination sequences.

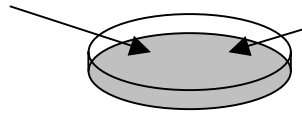
The final step in the generation of a pBC-based stx_{2d2} promoter::*lacZ* reporter plasmid was excision of the stx_{2d2} promoter::*lacZ* fusion fragment from pQF50.1 by digestion with *Bg*lII and *Sca*I (*Sca*I yields blunt-ended restriction fragments), and ligation into pBCterm. The vector with the terminator sequences (pBCterm) was linearized with *Bam*HI, treated with Klenow to make a blunt-ended fragment, and digested with *Bgl*II to provide suitable ends into which the (*Bgl*II/*Sca*I) promoter::*lacZ* fusion could be ligated (Fig. 3C). The resulting plasmid, that consisted of the stx_{2d2} reporter cassette ligated into pBCterm, was named pLTRC and transformed into DH5α .

1. **Spread donor and recipient inocula on non-selective agar and incubate for 3 hours.**

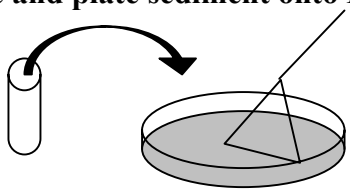
Donor: *E. coli* S17-1λ*pir*
+suicide vector **pATM161**(Km^r, Am^r)
100µl overnight culture

+

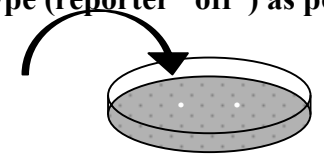
Recipient: DH5α Nal^r
+reporter **pLTRC** *stx*$_{2d2}$ promoter::*lacZ* (Cm ^{r})
100µl overnight culture



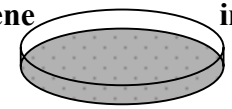
2. **Harvest mating mixture in saline and plate sediment onto Nal Cm Km LB agar with X-gal.**



3. **Isolate colonies with white phenotype (reporter "off") as potential activator mutants.**



4. **Extract plasmid DNA from mutants and retransform pLTRC into DH5α with chloramphenicol selection. Blue colonies with X-gal show that *lacZ* gene in reporter is not the site of mutation.**



5. **Cure mutants of reporter and transform mutants with Stx2d2 toxin gene clone (pSQ545).**

6. **Check for reduced cytotoxicity of pSQ545 in the DH5α mutants compared to DH5α wild-type.**

Figure 4. Flow diagram for transposon mutagenesis to identify a potential activator of Stx2d2 expression in DH5α.

These transformants produced blue colonies on chloramphenicol agar in the presence of X-gal.

2. Introduction and identification of transposon mutations in strain DH5α. The protocol used for transposon mutagenesis, adapted from deLorenzo, *et al.* (De Lorenzo *et al.*, 1990), is summarized in Figure 4. *E. coli* strain S17-1λ*pir* served as the conjugational donor for the delivery of the suicide vector pATM161 (graciously provided by A. Maurelli) into DH5α that had been transformed with pLTRC. Plasmid pATM161 contains a pi-protein-dependent R6K origin of replication, a beta-lactamase gene, and a mini-Tn*5* that encodes a kanamycin resistance gene (Km) and RP4 mobilization factor (De Lorenzo *et al.*, 1990). This plasmid replicates freely in a λ*pir*-lysogenized host but once it has been transferred into a *pir*$^-$ background, the vector is lost. Growth of recipients on kanamycin-containing medium selects for those in which the mini-Tn*5* Km has moved from the suicide vector to the host chromosome. The insertion of the transposons into the genes of the host results in random mutations by inactivation of the wild-type genes.

For mating experiments, equivalent densities of log phase cultures of S17-1λ*pir* transformed with pATM161 (donor) and DH5α transformed with pLTRC (recipient) were prepared. LB agar plates without antibiotics but supplemented with 1.0% sodium citrate (to reduce the incidence of transduction of λ*pir* to recipient cells) were inoculated with 100 μl of recipient cell suspension spread uniformly with a glass rod followed by 100 μl of the donor cell suspension. Plates were incubated 1 to 3 hours at 37^{o}C. The mating times were considerably shorter than those suggested by DeLorenzo, *et al*.(De Lorenzo *et al.*, 1990) because the recipient *E. coli* strain readily acquired λ*pir* if

incubated with the donor cells for more than three hours; such an event would negate the suicide vector strategy. Bacterial growth was collected from the agar surface by the addition of 2 ml of sterile saline to each plate that was stirred gently with a bent glass rod. These cell suspensions in saline were transferred to microcentrifuge tubes, and bacterial cells were harvested by centrifugation. The pelleted bacteria were then plated on X-gal LB medium that contained chloramphenicol (to retain pLTRC), naladixic acid (since DH5α is inherently resistant and the donor S17-1 cells are not), and kanamycin for the selection of transposon insertions. White colonies were presumed to have mutations that negatively influenced beta-galactosidase expression under direction of the stx_{2d2} promoter region. Plasmid DNA was isolated from prospective mutants and transformed into fresh DH5α under chloramphenicol selection to verify that an insertional mutation had not occurred in the *lacZ* reporter gene. Putative mutants were also subcultured onto ampicillin LB agar to determine whether they had acquired ampicillin resistance, an observation that would indicate that pATM161 had replicated through the acquisition of λ*pir*.

Putative stx_{2d2} activator mutants of DH5α were considered to be those colonies that consistently exhibited a white phenotype after repeated subcultures on X-gal LB agar, were ampicillin sensitive, and contained functional reporter plasmids (as described above). These mutants were then cured of the reporter plasmid. This curing of pLTRC was accomplished by repeated passages of the mutants in the absence of chloramphenicol. Chromosomal DNA was extracted from mutants that failed to grow in the presence of chloramphenicol and digested to completion with *Eco*RV. The DNA was

then probed with the *cat* DNA by Southern blot analysis (described above) to confirm the loss of the reporter plasmid.

To locate the site of mini-Tn*5* Km insertions in various fragments of the putative activator mutant DNA, additional digests of chromosomal DNA were done with *Eco*RV, *Xmn*I, *Sac*II, and *Nde*I and probed by Southern blot with the kanamycin gene. Three mutant strains that could not have arisen as siblings, that showed only one mini-Tn*5* Km insertion, and that inserted into distinct sites as evaluated by Southern blots generated from the four different digests were selected for further identification.

The genes into which the mini-Tn*5*s had inserted were determined by sequencing the DNA flanking the transposons. First, the mutations were cloned from restriction-digested chromosomal DNA fragments that had been separated by gel electrophoresis. Bands were excised that corresponded in size to the insertion sites previously identified in Southern blots probed with the kanamycin gene. Specifically, mutation 4 was located on a 3 to 4 kb *Sac*II fragment, mutant 31 on a 5 to 6 kb *Eco*RV band, and mutant 38 on a 4 to 5 kb *Eco*RV band. The fragments were eluted from the agarose gel and ligated into pKS$^-$ that had been digested with *Sac*II or *Eco*RV and treated with alkaline phosphatase. The ligation products were transformed into competent DH5α cells that were then plated onto kanamycin LB agar to select clones with mini-Tn*5* Km insertions. Primers were designed to sequence outward from the transposon into the flanking genes. The resulting sequences were subjected to BLAST comparison with the *Escherichia coli* K-12 genome (Blattner *et al.,* 1997). The presence of homologous genes in B2F1 was determined by PCR amplification of the genes in question using primers derived from the K-12

sequence. The products were cloned into pCR2.1 and sequenced to determine the extent of their homology to their K-12 homologues.

3. Determination of the influence on Stx2d2 expression of the transposon mutations in strain DH5α. Transposon mutants of DH5α that yielded white colonies on X-gal LB agar in the presence of the *stx*$_{2d2}$::*lacZ* reporter plasmid were postulated to contain mutations in DH5α chromosomal genes that influenced toxin expression in DH5α. To assess the actual effect of these mutations on toxin expression, mutants that contained mini-Tn*5* insertions determined to be in unique chromosomal sites (based on Southern blots of four different chromosomal restriction digests) were transformed with plasmids that harbored cloned toxin genes, i.e. pSQ547 (*stx*$_{2d2}$), pSQ545 (*stx*$_{2d2}$), pMJ100(*stx*$_2$), and pSQ543 (*stx*$_{2d1}$). The cytotoxicities to Vero cells of bacterial lysates from the transformed mutants were compared to those observed in parallel assays of bacterial extracts from wild-type DH5α transformed with the same toxin clones. Bacterial lysates of the mutants and wild-type DH5α were also assayed for toxin protein expression by Western blots that were probed with anti-Stx2 monoclonal antibody (assays done by Edda Twiddy). The mutants were also transduced with bacteriophage 933W to assess cytotoxicity in the DH5α mutants of a related toxin gene in single copy on the bacterial chromosome.

To address the possibility that the mutations exerted a generalized effect on plasmid copy number or expression of plasmid-borne genes non-specifically, the DH5α mutants and wild-type were transformed with Bluescript vectors pKS and pBC alone, and beta-galactosidase expression was measured. Mutant and wild-type DH5α were also

transformed with an unrelated *Escherichia coli* gene, *tir*, in pKS$^-$ and protein concentrations were compared by Western blot with the assistance of Dr. James Sinclair.

The *caiD*, *caiE*, *caiDE*, and *ycdU* genes of DH5α were amplified by PCR and ligated into the low copy number vector pWKS130 (Wang and Kushner, 1991) into which a *cat* gene had been ligated at the *Bam*HI site. The modification of antibiotic resistance markers was necessary because these clones were used for complementation assays in DH5α that contained Km^r mutations and toxin gene-encoding plasmids that contained ampicillin resistance markers.

C. Introduction of Tn*5* mutations into strain B2F1 by allelic exchange. To determine whether the mutations in DH5α that were associated with reduced expression of cloned toxin genes had a role in toxin expression in B2F1, the DH5α mutations were moved into B2F1 by allelic exchange. The mutations had previously been cloned from DH5α into pKS$^-$ for DNA sequencing of the insertion sites. This sequencing revealed the presence of adequate flanking DNA for the homologous recombination step required during allelic exchange. The mutated DNA fragments in mutants 31 and 38 were excised from pKS$^-$ with *Eco*RV to yield blunt ends; these ends allowed the mutant gene fragments to be cloned into the *Hin*cII site within the multiple cloning site of the suicide vector pSTAMP. Mutated DNA from mutant 4 was originally cloned from DH5α chromosomal DNA on a *Sac*II fragment. Since this fragment was difficult to clone, the transposon insertion was re-isolated from a 4 – 5 kb *Eco*RV chromosomal digest fragment (as identified by Southern blot). This *Eco*RV fragment with the mutated DNA from mutant 4 was then ligated into pSTAMP as described above. The introduction of

the DH5α mutations into B2F1 was done by the same strategy as described above for the generation of individual toxin mutations in B2F1 with the pSTAMP suicide vector.

IV. Bacteriophage Studies

A. Bacteriophage growth conditions and induction. Reduced salt (2.5 g/L) LB media supplemented with 10 mM $CaCl_2$ (hereafter called modified LB media) were used for bacteriophage induction and plaque detection. Bacteriophages were induced with 0.5 μg/ml mitomycin C (Sigma) that was added after broth cultures had been incubated for 1 hour. To test for antibiotic induction of bacteriophages, ciprofloxacin (Bayer, 25 ng/ml) or fosfomycin (Sigma, 800 ng/ml) was incorporated into the modified LB broth. Induced cultures were grown with aeration for 4 hours at 37°C. Bacteriophages were harvested by chloroform lysis of the host bacterial strain in suspension, centrifugation of the lysate, and filter sterilization (0.45 μm) of the resultant supernatant. This clarified cell supernatant was then serially diluted in ten-fold increments in LB broth. Samples (100 μl) of each dilution were incubated at 37° C for 20 minutes with 200 μl of log-phase indicator cells. These phage-bacterial cell cultures were then added to 2.7 ml of warm, liquid modified LB top agar, the mixtures overlaid onto LB agar in petri dishes, and the top layer permitted to solidify at room temperature (double-layer method). After overnight incubation of these double layer plates at 37° C, the top agar was examined for plaques.

B. Isolation and identification of *stx*$_{2d1}$ lysogen. Cultures of wild-type B2F1 were induced with mitomycin C and grown for four hours. The cells were then treated with 0.5 ml chloroform per 3.0 ml of broth culture, and the resulting lysate was used without

dilution to infect indicator strains C600, 395-1 and DH5α. The bacterial cell-phage mixture was suspended in soft top agar and plated as described above. Samples of the surface agar that contained plaques were excised, suspended in broth, emulsified, and the supernatant diluted 10^{-6}-fold. A 100 μl sample of the diluted broth was plated onto agar and incubated overnight. Isolated colonies that appeared were subcultured onto LB agar and were transferred onto nitrocellulose membranes to be screened for toxin gene acquisition. Potential lysogens were identified from the colony blots by hybridization with an *stx*$_{2d1}$ toxin gene probe.

Isolates of DH5α that were *stx*$_{2d1}$ probe-positive were transformed with a clone of the *recA* gene (pIM10 generously provided by T. Oelschlaeger and J. Hacker) to complement the *recA* defect in DH5α (Fuchs *et al.*, 1999). The putative lysogens were grown in broth with and without addition of mitomycin C, and the cultures then tested for Vero cell cytotoxic activity. The toxin gene transduced into DH5α was amplified by PCR with primers LT2 or LT10 and JCS2 from chromosomal DNA of the lysogen. To verify that *stx*$_{2d1}$ and not *stx*$_{2d2}$ had been acquired, the resulting PCR product was digested with *Eco*RV and *Acc*I and the resulting fragments compared in size to fragments obtained by corresponding restriction digests of PCR products derived from *stx*$_{2d1}$ and *stx*$_{2d2}$ clones.

C. Electron Microscopy. Five hundred milliliter cultures of C600(933W), B2F1 mutant 7-4, and the DH5α lysogen transformed with pIM10 were induced with mitomycin C, incubated for 4 hours, and the cellular material removed by centrifugation. Bacteriophages were collected from the supernatant by precipitation with polyethylene glycol 8000 (PEG). Chloroform was used to extract the PEG and cell debris, and bacteriophages were harvested from the aqueous phase by centrifugation as described

(Sambrook *et al.*, 1989). The bacteriophage pellet was resuspended in SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris·Cl, pH 7.5, 0.01% gelatin) with gentle agitation at $4^{O}C$ overnight. Approximately 15 μl of the suspension was applied to Formvar-coated copper grids (Ladd Industries, Burlington, VT). After 20 minutes, excess liquid was absorbed from the edges of the grids with a paper towel and 15 μl of 2% uranyl acetate (Sigma) was applied to the grids for negative staining. Excess stain was removed by absorption as above. The dried grids were viewed in a Philips electron microscope Model CM100 under 94,000X magnification. The C600(933W) preparation served as a positive control for the phage purification and staining procedures.

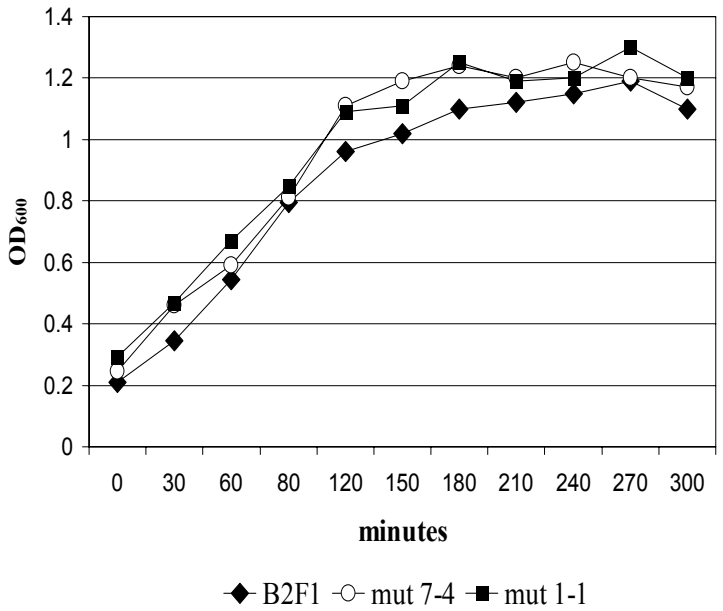
RESULTS

I. Characteristics of the individual toxin alleles of strain B2F1

A. Cytotoxicity and virulence of the strain B2F1 toxin mutants. Mutants of *E. coli* strain B2F1 were generated in which either stx_{2d1} or the stx_{2d2} gene was disrupted (Fig. 1). The individual mutants grew at the same rate as wild-type B2F1 (Fig. 5A). The single toxin-producing mutants of B2F1 did not produce equivalent levels of cytotoxin. Rather, the geometric mean CD_{50}/ ml of sonically disrupted broth cultures of the Stx2d1-producing mutant was approximately nine-fold lower than wild-type B2F1 (Fig. 5). Conversely, the Stx2d2-producer produced essentially the same levels of cytotoxin as the wild-type. When the two mutants were compared, a 7-fold difference in geometric mean CD_{50}/ ml of sonically disrupted broth culture was noted (Fig. 5B). Although the 95% confidence intervals of the geometric means of the groups overlapped, paired comparisons of these mutants in different experiments always showed that the Stx2d1-producing mutant was less cytotoxic than the Stx2d2-producing mutant. In contrast, when the individual stx_{2d1} and stx_{2d2} genes were separately ligated into the same type vector, the clones expressed comparable levels of toxin as determined by the Vero cell cytotoxicity assay (Lindgren, 1993). This latter result, combined with the lower toxicity of the mutant that produced Stx2d1 suggests that Stx2d1 expression is repressed in B2F1.

The toxicity difference between the mutants was even more pronounced *in vivo*, as measured in comparative lethal dose studies in mice. The oral LD_{50} of wild-type B2F1 in streptomycin-treated mice was less than 20 CFU. The Stx2d2-producing mutant was still highly virulent ($LD_{50} = 2 \times 10^2$ CFU), but the Stx2d1-producing mutant was almost

Panel A.



Panel B.

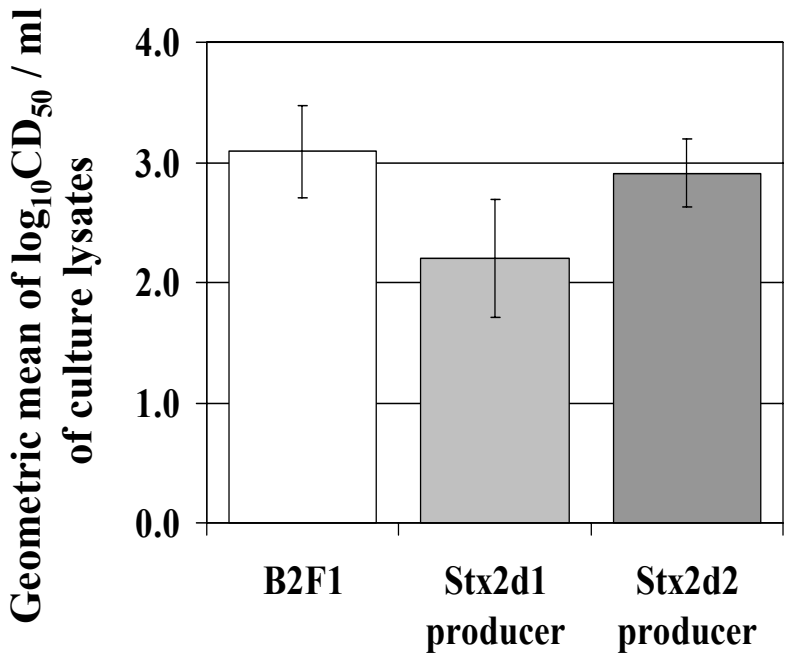


Figure 5. Comparison of strain B2F1 and its single toxin-producing mutants.
Panel A. Growth kinetics. Overnight cultures were diluted 1:100 in LB broth and incubated at 37°C with shaking. OD_{600} was measured at each time point**.** Mutant 7-4 produces only Stx2d1 and Mutant 1-1 produces only Stx2d2. (Results of one experiment)

Panel B. Cytotoxicity. Sonicated lysates of overnight cultures were used to inoculate Vero cells.Columns represent geometric means of seven or eight experiments, and error bars represent the 95% confidence intervals for each group.

completely attenuated ($LD_{50} = 10^8$ CFU). These results further support the hypothesis that the individual toxin genes are intact but differentially regulated in B2F1 and that Stx2d2 contributes more to cytotoxicity and pathogenicity in mice than does Stx2d1.

B. Comparison of DNA sequences flanking *stx*$_{2d1}$ and *stx*$_{2d2}$. Previous attempts in this laboratory to induce bacteriophages from B2F1 were not successful (Lindgren, 1993). Therefore, I initially examined the DNA sequence directly upstream of both *stx*$_{2d1}$ and *stx*$_{2d2}$ to identify other possible regulatory mechanisms that might influence the differential expression of either toxin. No obvious transcriptional regulatory elements were detected. Instead, I found sequences homologous to lambdoid bacteriophage genes upstream of both toxin genes. I continued to sequence 4 kilobases (kb) upstream and downstream from each *stx*$_{2d}$ gene and compared the sequences flanking *stx*$_{2d1}$ to those flanking *stx*$_{2d2}$ (Fig 6). The DNA sequences upstream of each were very similar to one another (95% identical) over a distance of 1.9 kb. This homologous region contained three putative transfer RNA genes, *ileZ*, *argN*, and *argO,* directly upstream of each toxin gene and a putative DNA methyl transferase gene just upstream of the tRNA genes. There was a complete open reading frame for the putative methyl transferase upstream of *stx*$_{2d2}$, whereas the methyl transferase gene upstream of *stx*$_{2d1}$ contained an internal stop sequence that would result in a truncated protein product. Upstream beyond the methyl transferase genes the sequences diverged and shared no significant homology.

Downstream of both *stx*$_{2d1}$ and *stx*$_{2d2}$ I detected an ORF homologous to *yjhS* and of comparable size (1 kb) to the K-12 gene. The function of the *yjhS* product has not yet been defined. The *stx*$_{2d}$ genes were followed further downstream by sequences homologous to the lambdoid bacteriophage holin gene, *S.* The intervening DNA

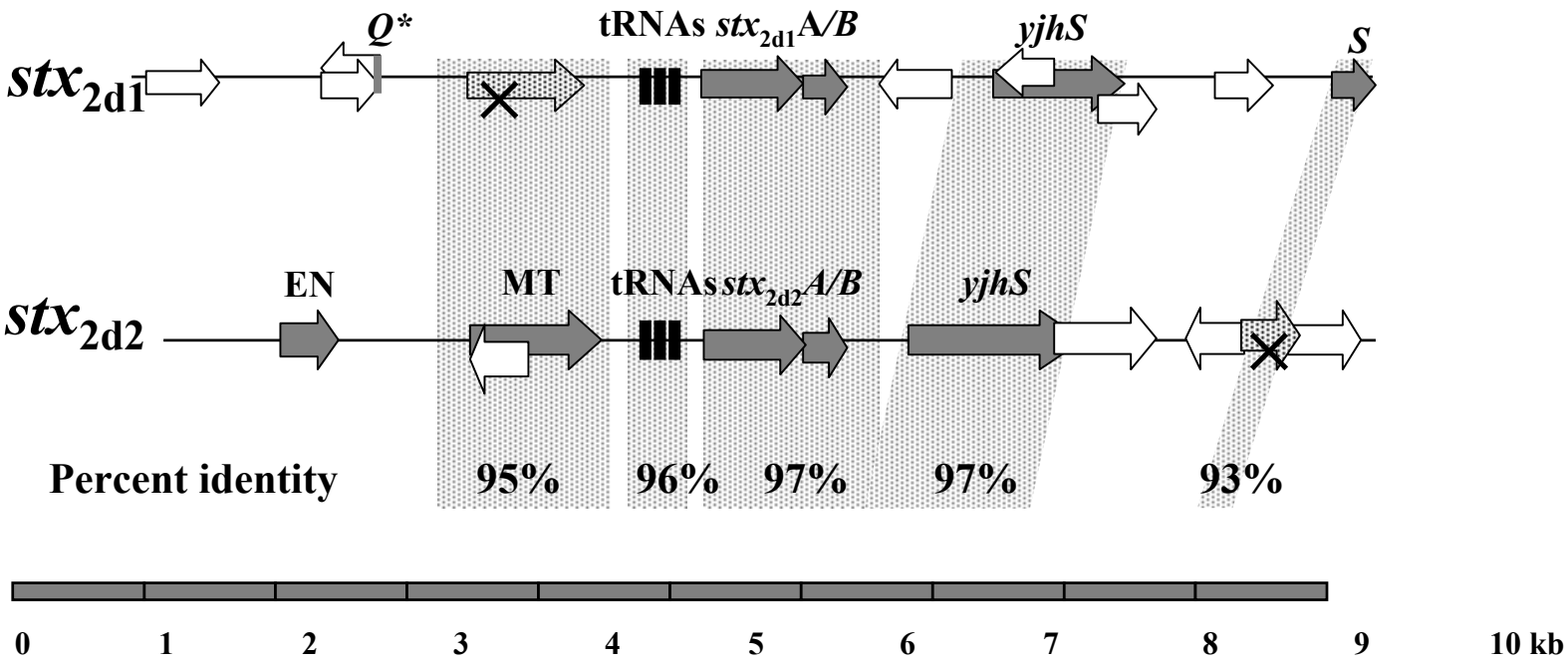


Figure 6. Comparison of the DNA sequences flanking stx_{2d1}- and stx_{2d2}. Arrows show relative length and direction of open reading frames. Gray arrows represent ORFs with homologues in GenBank. White arrows indicate no homologues were identified. The regions of greatest DNA sequence similarity between the stx_{2d}-associated sequences appear on stippled background. Abbreviations for putative genes are, EN, endonuclease, MT, methyl transferase, *Q*,* 50bp fragment homologous to the 5' end of Q gene sequence in 933W, tRNAs, *ileZ, argN, argO, S,* holin lysis gene, *yjhS, E. coli* K-12 homologue with unknown function. ORFs containing × encode proteins truncated by stop codons.

sequences between the toxin genes, the *yjhS* homologues and the holin genes were less than 75% identical. In sum, the genetic arrangement and DNA sequence of the stx_{2d1}- and stx_{2d2}-flanking regions were very similar from approximately 2 kb upstream to 4 kb downstream of the toxin genes, a finding that suggests they share a common origin. Because of the similarity of these DNA sequences, I could not predict a mechanism for the differential expression of stx_{2d1} and stx_{2d2} in B2F1.

C. Similarity of the stx_{2d}–flanking DNA to other toxin-converting phages. Next the genetic organization and DNA sequences of the stx_{2d}-flanking regions to other toxin-converting phages and DNA sequences in GenBank were compared (Fig. 7). Stx2 toxin-converting phages encode tRNA genes immediately upstream of their toxin genes, and the transcriptional antiterminator-encoding *Q* gene is directly upstream of the tRNA genes (Schmidt *et al.*, 1997b; Plunkett III *et al.*, 1999). In contrast, both stx_{2d} genes had sequences homologous to a bacteriophage-associated DNA methyl transferase gene (further downstream than the homologue in stx_2-converting phages) and no *Q* open reading frame upstream of the tRNA genes. Furthermore, I did not identify a *Q* gene homologue within the 4 kb that were sequenced upstream of either toxin allele. However, I observed faint bands on Southern blots of B2F1 restriction-digested chromosomal DNA (both *Bam*HI and *Hind*III restricted) probed with a 430 bp Q gene fragment that was PCR-derived from C600(933W) (Fig. 8A) . Additionally, I saw a strongly hybridizing band when I used the same fragment to probe a digest of the cosmid clone that contains stx_{2d1} (pSQ12), but no such band was evident on a similar digest of the stx_{2d2} cosmids (pJES210 and pJES54) so probed (Fig. 8B). The observed Q gene hybridization signal

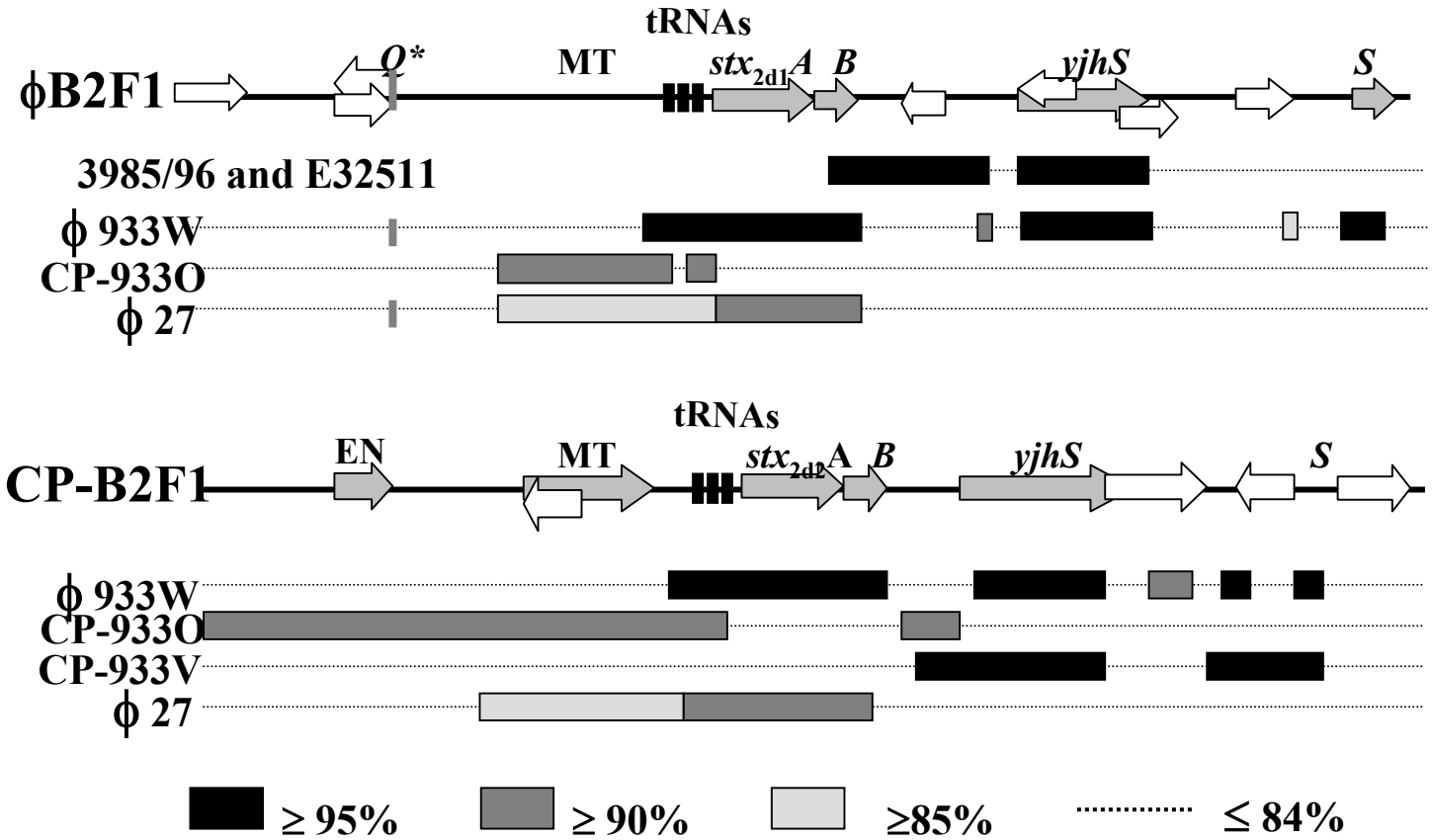


Figure 7. Comparison of the DNA flanking *stx*$_{2d1}$ and *stx*$_{2d2}$ with the most closely related sequences in GenBank. Abbreviations are the same as those used in Figure 6. CP, cryptic phage (*stx*$_{2d2}$-associated).

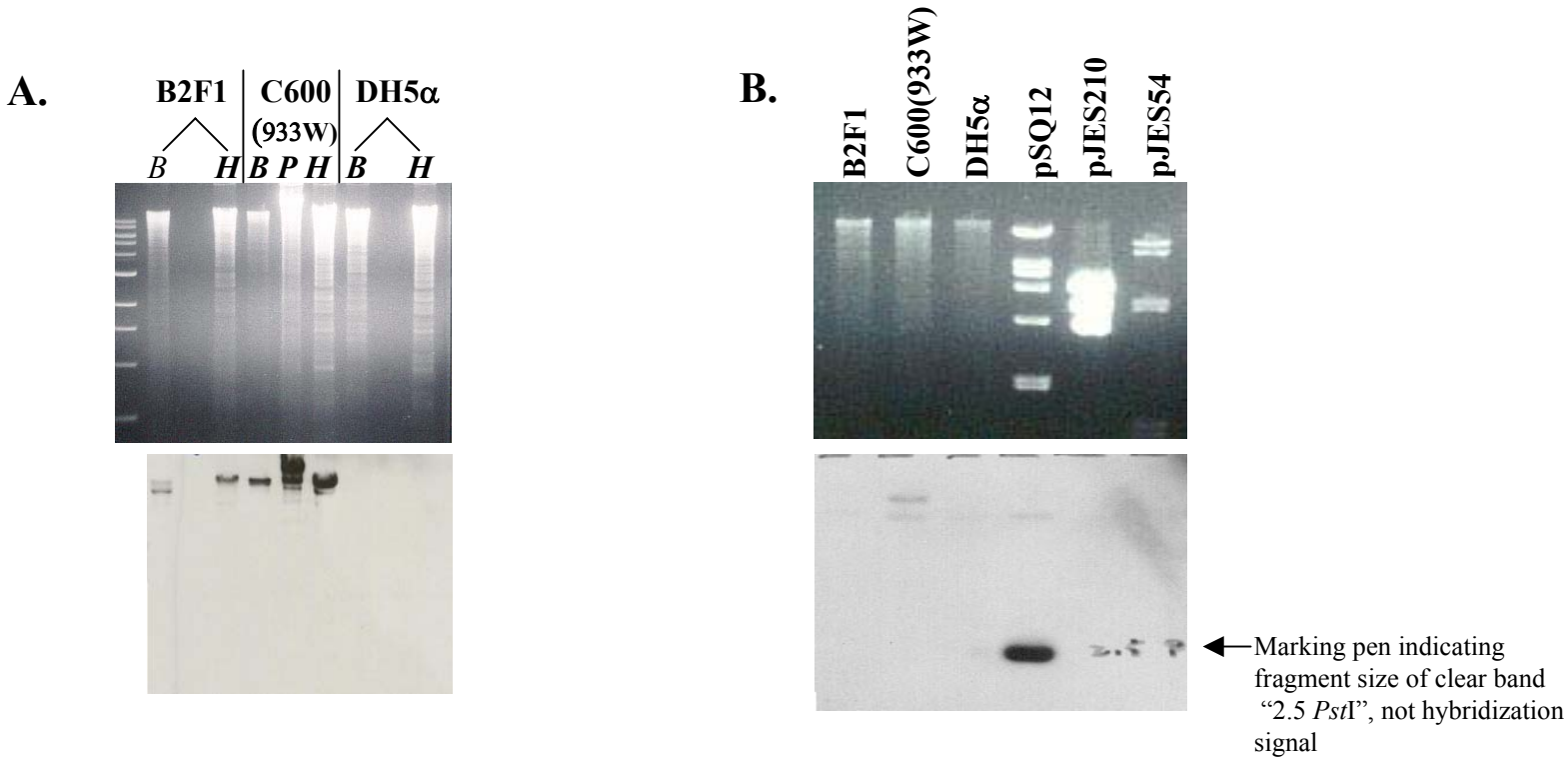


Figure 8. DNA digests probed with the Q gene of ϕ933W. Panel A. Agarose gel of chromosomal DNA digested with *B, Bam*HI, *H, Hind*III, or *P, Pst*I , probed with the ECL-labeled Q gene probe below. Panel B, *Pst*I digest of chromosomal DNA and cosmid DNA including, pSQ12 (stx_{2d1}), pJES210 (stx_{2d2}) and pJES54 (stx_{2d2}) probed with the Q gene probe, below.

may correspond to a 50 bp sequence 3 kb upstream of *stx*$_{2d1}$ that was 90% identical to the 5' portion of the *Q* gene from the *stx*$_2$-bearing bacteriophage 933W (Plunkett III *et al.*, 1999). This observation suggests that a Q gene homologue may have once existed in that region (Fig. 7).

DNA sequences upstream of both *stx*$_{2d}$ alleles most closely resembled the non-toxin-bearing cryptic phage (CP) CP-933O of strain 933EDL (Perna *et al.*, 2001) and of the Sakai strain of *E. coli* O157:H7 (Hayashi *et al.*, 2001)(Fig. 7). Specifically, neither the sequences upstream of *stx*$_{2d1}$ and *stx*$_{2d2}$ nor the region upstream of CP-933O encode a homologue of the 933W *Q* gene. Furthermore, the DNA sequence 5' of *stx*$_{2d2}$ was homologous to CP-933O over the entire 4 kb sequenced. The comparable region of *stx*$_{2d1}$ was homologous to CP-933O for 1.9 kb and diverged from CP-933O at the same site where the upstream *stx*$_{2d2}$ and *stx*$_{2d1}$ sequences also began to differ. The region up to 1.9 kb upstream of each toxin gene was also 89% identical to the corresponding region in the *stx*$_{2e}$-bearing phage ϕ27 (Muniesa *et al.*, 2000). Beyond the point at which the *stx*$_{2d1}$-flanking sequence diverged from *stx*$_{2d2}$ one ORF was identified that was homologous, in part, to a putative cytoplasmic protein, "STM2240" in *Salmonella typhimurium* (McClelland *et al.*, 2001).

The regions downstream of *stx*$_{2d1}$ and *stx*$_{2d2}$ have an organizational structure like that of *stx*$_1$- and *stx*$_2$-bearing phages (Unkmeir and Schmidt, 2000), both of which also encode an ORF homologous to the *yjhS* gene of *E.coli* K-12 (albeit larger, 2 kb). The sequences downstream of the two Stx2d genes showed some highly conserved regions between them, but overall they resembled different toxin-associated phages (Fig. 7). The region downstream of *stx*$_{2d1}$ most closely resembled the corresponding regions

downstream of *stx*$_2$ in *E. coli* strain 3985/96 and *stx*$_{2c}$ in strain E32511 (Unkmeir and Schmidt, 2000) and was similar, but less so, to the region downstream of *stx*$_2$ in 933W. The sequence 3' of *stx*$_{2d2}$ most closely resembled the *stx*$_1$-bearing cryptic phage of strain EDL933, CP-933V but also shared regions of strong identity to phage 933W. Although the DNA 5' to both of the toxin genes was similar to ϕ27, neither of the *stx*$_{2d}$ toxin genes showed homology to ϕ27 in the 3' direction. In sum, the *stx*$_{2d}$-flanking regions resembled other toxin-converting phages in organization, but these DNA sequences did not show strong identity to any one of the previously described phages. Rather the sequences surrounding the *stx*$_{2d}$ alleles appeared as a patchwork of strong homologies with a variety of specific genes associated with both inducible and cryptic phages.

II. Bacteriophage studies in *E. coli* strain B2F1

A. Results of bacteriophage induction. As a first step to determine whether an inducible, toxin-converting phage was indeed present in strain B2F1 (as implied by the sequence data), the cytotoxicity of supernatants from each toxin mutant was measured after growth in the presence or absence of mitomycin C. Although the broth cultures treated with mitomycin C remained relatively turbid, the levels of cytotoxicity of wild-type B2F1 and the Stx2d1-producing mutant were greatly enhanced by exposure to mitomycin C. The cytotoxicity of the Stx2d2-producing mutant was unchanged by mitomycin C treatment (Fig. 9). These results suggested that Stx2d1, but not Stx2d2, is associated with an inducible bacteriophage. Tiny turbid plaques were observed on *E. coli* strains 395-1, C600, and DH5α that were treated with cell lysates from either toxin

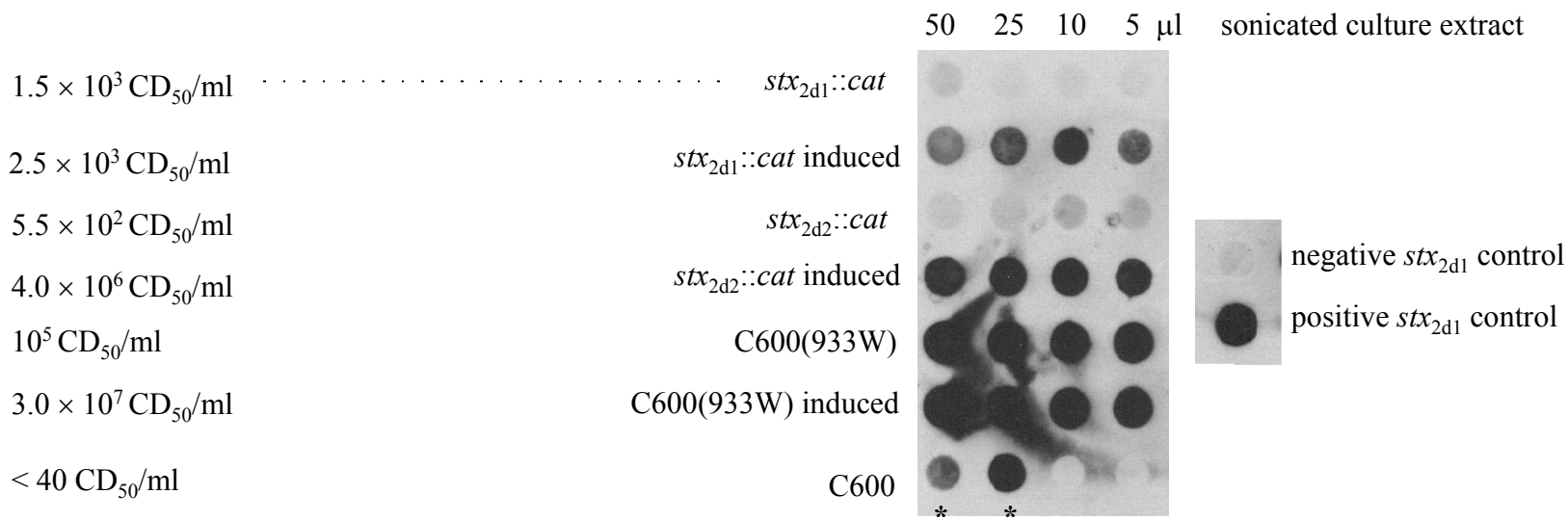


Figure 9. Effect of mitomycin induction on toxin gene concentration in the single toxin mutants of B2F1 and C600(933W). Culture lysates of the B2F1 toxin mutants and C600(933W) were grown with or without mitomycin C induction**.** Toxin gene concentration was determined by dot blot hybridization with an ECL-labeled stx_{2d1} probe. Plasmid DNA (on right) with and without target sequences served as controls for probe. Cytotoxicity of the same batch of culture lysates was assayed. C600 does not encode any Shiga toxin genes. It is likely that the positive signal in the C600 wells (*) resulted from leakage from C600(933W).

mutant. Because the plaques were barely discernable, it was not possible to determine the actual numbers of plaque-forming units; however the most concentrated samples applied to the host bacterial lawn resulted in a very mottled appearance to the plate surface, and this effect was eliminated by dilution of the phage inoculum.

Dot blots of cell supernatants of both mutants probed with the *stx*$_{2d}$ gene revealed an increase in gene dosage upon mitomycin C treatment (Fig. 9). However, the probe DNA used to detect *stx*$_{2d}$ in these dot blots could not differentiate between mutant and wild-type toxin alleles. When the dot blots were probed with the chloramphenicol acetyl transferase gene DNA to distinguish the mutated from the functional toxin allele, only the mutated *stx*$_{2d1}$::*cat* copy number was increased with induction (Fig 10). Mitomycin C induction increased *stx*$_{2d1}$ and *stx*$_{2d1}$::*cat* gene dose and cytotoxicity in the B2F1 mutant with an intact Stx2d1 gene, while Stx2d2 gene copy number and expression were unchanged by this treatment. These findings provide further evidence that *stx*$_{2d1}$ expression is bacteriophage-associated but *stx*$_{2d2}$ is not in strain B2F1.

B. Phage immunity studies. The DNA sequence immediately upstream of each *stx*$_{2d}$ was 95% identical to the Stx2 toxin-converting phage 933W. This raised the question of whether the putative *stx*$_{2d1}$-bearing phage shared lysogenic immunity with phage 933W. For this purpose, Clare Schmitt tested the capacity of lysates from B2F1, the individual toxin-producing mutants, and a C600 lysogen of 933W to form plaques on one another (unpublished data). The results are summarized in Table 4. Tiny plaques were observed on C600 and C600(933W) co-cultured with extracts from B2F1 or either of the individual toxin-producing mutants of B2F1, a finding that indicates that the phage released from B2F1 were not of the same immunity group as 933W. As expected, the B2F1 phage

Concentration of mutated toxin genes of B2F1

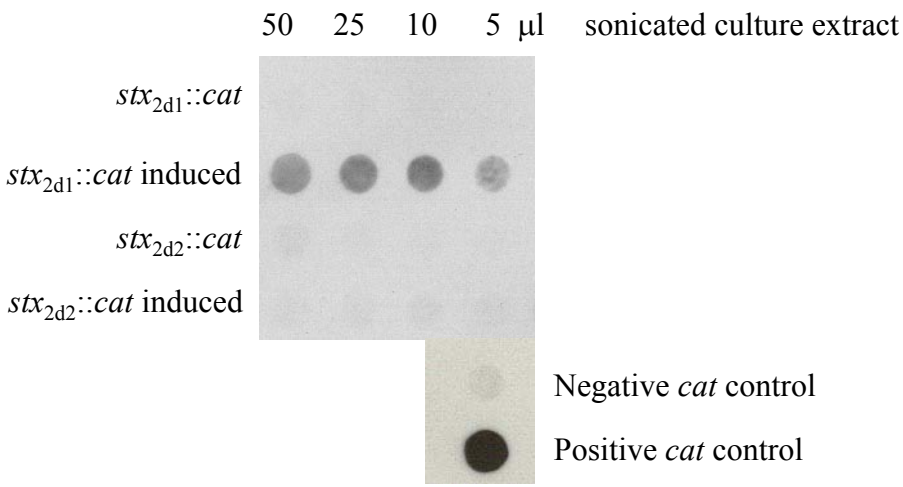


Figure 10. Effect of mitomycin C induction on *cat* gene concentration in the single toxin mutants of B2F1. This blot is duplicate of the blot shown in Fig. 9 prepared at the same time with the same cell lysates.The blot was probed with *cat* to compare gene copy number of mutant alleles (with *cat* insertions) associated with mitomycin C induction. Plasmid DNA with and without target genes served as positive controls.

Table 4. Plaque formation following infection of host strains with chloroform-treated culture supernatants from B2F1, single toxin-producing mutants, a phage negative control strain, and an Stx2-converting phage lysogen.

Host strains of *E. coli*	Strain from which phage lysate was prepared for challenge of host cells				
	C600	B2F1 wild-type *stx*$_{2d1}$* *stx*$_{2d2}$	B2F1 mutant *stx*$_{2d1}$* *stx*$_{2d2}$::*cat*	B2F1 mutant *stx*$_{2d1}$::*cat* * *stx*$_{2d2}$	C600(933W)
C600	-	+	+	+	+
B2F1	-	-	-	-	-
C600 (933W)	-	+	+	+	-

(+) plaques observed, (-) no plaques observed, (*) phage-borne toxin allele

lysates failed to produce plaques on B2F1, and lysates of C600(933W) did not produce plaques on C600(933W). Although we also anticipated that lysates from C600(933W) would produce plaques on strain B2F1 because lysogenic immunity is generally reciprocal, no plaques were observed. Other factors, such as capsule type or lack of appropriate receptor molecules, may have prevented infection of B2F1 with 933W. It is also possible that strain B2F1 harbors another bacteriophage of the same immunity group as phage 933W.

C. Influence of ciprofloxacin on toxin expression. Quinolone antibiotics induce bacteriophages and increase toxin production in strains that harbor Stx2-bearing bacteriophages (Matsushiro *et al.*, 1999; Zhang *et al.*, 2000). The investigators who reported this finding speculated that the inhibitory effect of these antibiotics on DNA gyrase probably results in the accumulation of single-stranded DNA fragments that trigger the SOS response and a subsequent RecA-mediated conversion of the phage from the lysogenic to the lytic cycle. Based on the observation of quinolone-mediated induction of Stx2-expressing phage, I decided to test whether subinhibitory concentrations of ciprofloxacin exert a similar inductive effect on Stx2d1 expression *in vitro* and in infected animals. First, I compared cytotoxicity of the single toxin-producing mutants grown in broth alone to those grown in broth supplemented with mitomycin C, ciprofloxacin, or fosfomycin, an antibiotic that does not induce stx_2-converting phages from STEC O157:H7 (Zhang *et al.*, 2000). The results of those antibiotic studies are summarized in Fig. 11. Ciprofloxacin induced Stx2d1 production nearly as well as mitomycin C, while fosfomycin did not cause an increase in cytotoxicity from the mutants. As was the case with mitomycin C induction, increased cytotoxicity with

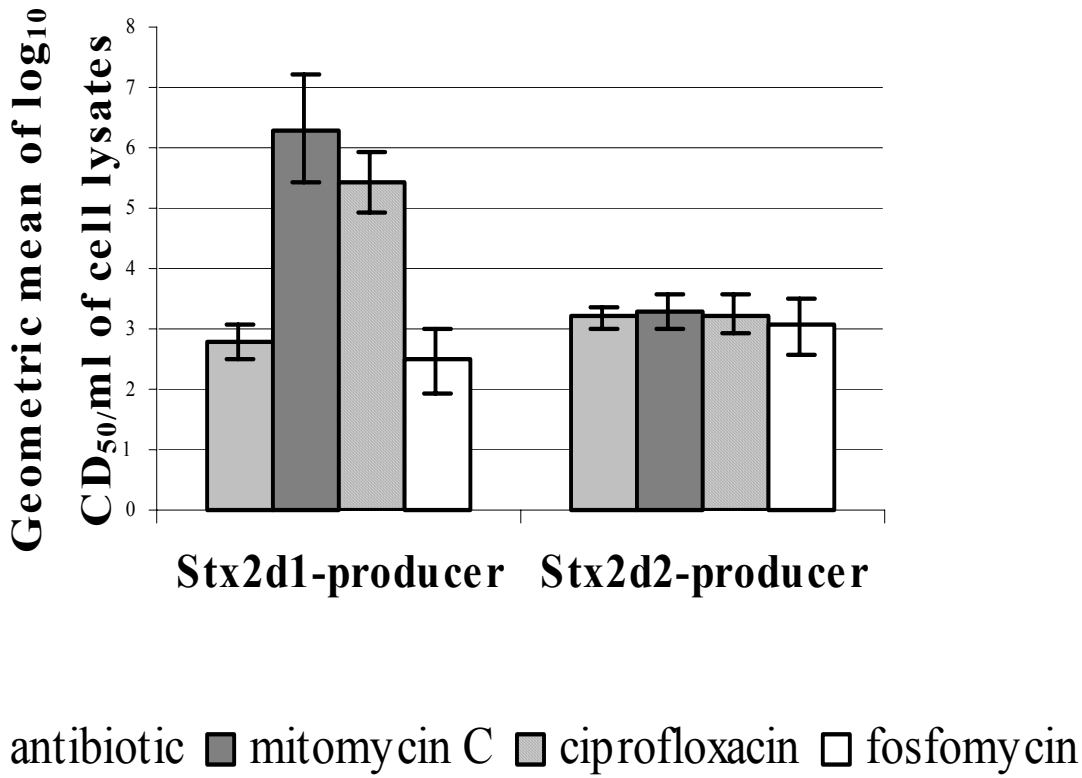


Figure 11. Cytotoxicity of single toxin-producing mutants of B2F1 treated with subinhibitory concentrations of agents that induce bacteriophages. Vero cells were inoculated with supernatants from sonicated lysates of overnight broth cultures. Columns represent $\log_{10}$ of geometric means of three experiments and error bars depict the 95% confidence intervals.

ciprofloxacin induction was only seen with B2F1 (not shown) or the mutant that carried a functional stx_{2d1} gene.

Next, I determined whether the enhanced toxicity of the Stx2d1-producer that I observed *in vitro* upon treatment with ciprofloxacin would render this strain virulent for mice treated with ciprofloxacin. The Stx2d1-producing mutant was fed to twenty mice and, after 48 hours, ten of the mice were treated with subclinical doses of ciprofloxacin according to a modified version of the protocol used to increase Stx2 production *in vivo* in antibiotic-treated mice (Zhang *et al.*, 2000). The remainder of the infected mice received intraperitoneal injections of PBS to serve as controls. None of the 10 infected mice that received PBS and none of the 5 uninfected mice given ciprofloxacin died. However, 6 out of 10 of the ciprofloxacin-treated mice infected with the Stx2d1-producing mutant died compared to 9 of the 10 control mice fed wild-type B2F1.

Fecal pellets from the infected mice were pooled by group and cultured to verify that the mice had become colonized. After antibiotics were administered, fecal pellets were tested for cytotoxicity and cultured for CFU/g feces. In the ciprofloxacin-treated group, the colony counts per gram feces decreased with treatment, as expected, while the CD_{50}/g feces increased sharply. In the PBS-treated controls the CFU/g remained relatively constant while the CD_{50}/g increased less dramatically during infection than for the ciprofloxacin-treated mice. The log values of CD_{50} per CFU for each group at each sample day are depicted in Fig.12. The ciprofloxacin-treated mice showed up to two-fold higher fecal toxin levels per CFU per g feces compared to the PBS-treated controls. The results of this mouse experiment strongly suggest that ciprofloxacin up-regulated toxin expression in the B2F1 mutant that only expresses Stx2d1. The implication of this

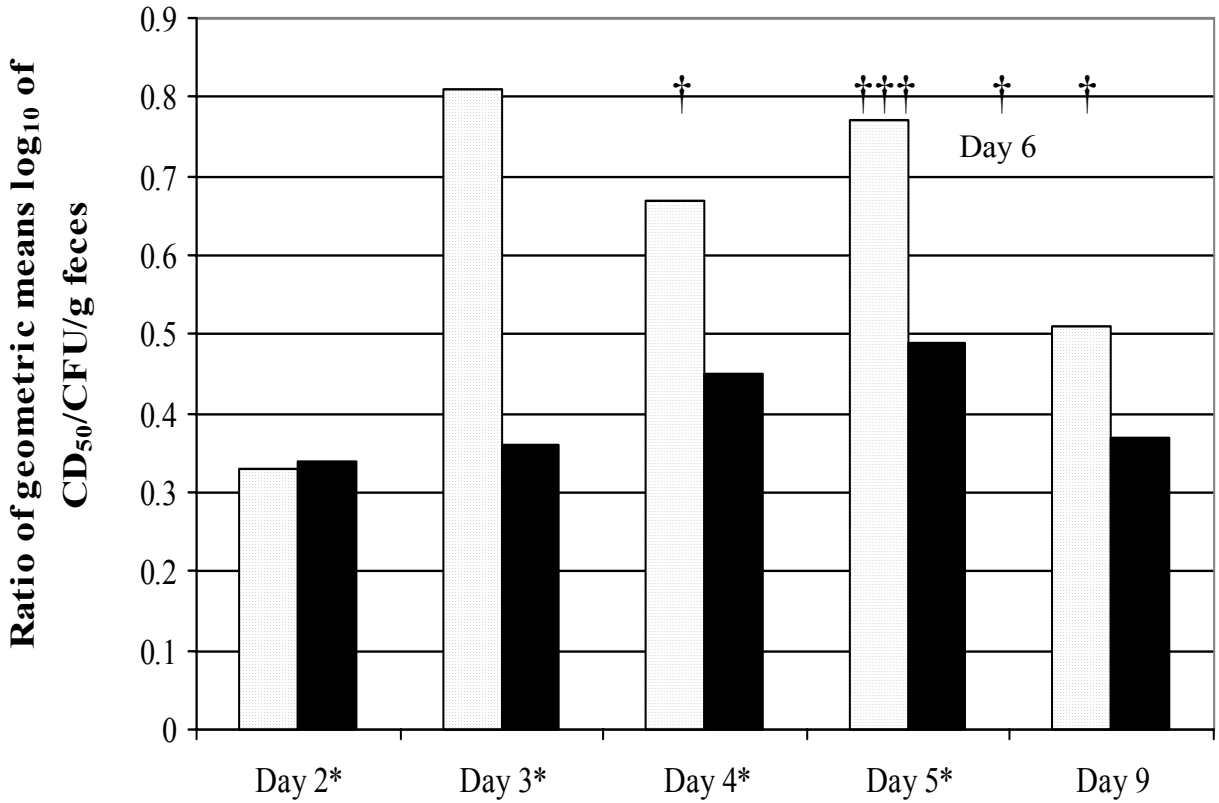


Figure 12. Ratio of $\log_{10}$ cytotoxicity (CD_{50}) to $\log_{10}$ bacterial counts (CFU) per gram feces from mice fed the Stx2d1-producing mutant of strain B2F1. Stippled columns represent samples from mice treated with ciprofloxacin, and black columns represent those treated with PBS. The ratio of cytotoxicity per CFU is calculated from $\log_{10}$ geometric means from two groups of 5 mice each in each treatment category. Asterisks (*) represent the days on which the animals were treated and (†) represent when the 6 animals died.

conclusion, in the context of the *in vitro* induction studies, is that ciprofloxacin therapy induced an Stx2d1-coverting phage *in vivo*.

D. Isolation of a strain DH5α lysogen encoding *stx*$_{2d1}$. The increased cytotoxicity and mouse virulence of the B2F1 mutant that produces Stx2d1 when exposed to ciprofloxacin, together with the formation of bacteriophage plaques when these mutant bacteria were induced with mitomycin C, suggested that Stx2d1 expression and production of phage(s) were coordinated. To assess whether the simultaneous increase in toxin expression and phage plaques occurred because *stx*$_{2d1}$ was actually borne on a phage, I attempted to transduce the toxin gene into other *E. coli* strains. Cultures of wild-type B2F1 were induced with mitomycin C, and sterile cell lysates were prepared and used to heavily infect C600 or 395-1. Samples of the plaqued soft agar were harvested and subcultured for potential lysogens. I reasoned that organisms which did not lyse when challenged with the B2F1 cell extract were either uninfected by phages or were lysogenized and therefore protected from lysis. To identify potential lysogens, I screened the colonies that I recovered from the cultured plaques by PCR for toxin genes or colony blot hybridization with a toxin gene probe. Despite the appearance of incomplete plaques on 395-1 and C600, repeated efforts failed to yield any lysogens of these RecA$^+$ strains. Therefore, we challenged DH5α with B2F1 phage lysates because that RecA$^-$ strain had been used successfully to isolate a lysogen of the *stx*$_{2e}$-bearing phage ϕ27 (Muniesa *et al.*, 2000). Of the 538 DH5α colonies screened, 38 reacted with the toxin gene probe. Chromosomal DNA of two representative probe-positive colonies was subjected to PCR with 2 sets of primer pairs that would yield internal toxin gene sequences of two different lengths. Fragments of the appropriate sizes for *stx*$_{2d}$ were obtained. To verify that the

stx$_{2d1}$ allele, and not the *stx*$_{2d2}$ allele had been transduced, the PCR products were treated with *Eco*RV that cleaves *stx*$_{2d2}$ but not *stx*$_{2d1}$ and *Acc*I that cuts once in *stx*$_{2d1}$ and twice in *stx*$_{2d2}$. The PCR products were resistant to digestion with *Eco*RV but cut with *Acc*I, yielding fragments consistent with digests of control PCR products obtained from an *stx*$_{2d1}$ clone and distinguishable in size from those obtained from a clone of *stx*$_{2d2}$ (Fig. 13).

To test whether a functional toxin gene had been transduced, I sought to demonstrate that the putative lysogens were cytotoxic. Preliminary Vero cell assays showed that the transductants were not cytotoxic. However, DH5α lacks the RecA protease necessary to cleave the phage repressor of late gene expression, a step that is required for the lytic cycle to be induced in lysogens that contain the lambda-like 933W phage and for concomitant Stx2 expression (Neely and Friedman, 1998; Fuchs *et al.*, 1999). Therefore, in an effort to promote expression of Stx2d1 from the putative lysogen, I complemented that lysogen of DH5α with a cloned *recA* gene contained in pIM10. These transformants produced 2.9×10^2 CD_{50} / ml of broth without mitomycin C induction and 10^4 CD_{50}/ml with induction, a finding that supports the hypothesis that Stx2d1 expression is linked to phage induction. Additionally, phage preparations derived from the induced RecA-complemented lysogen produced tiny plaques on DH5α and 395-1 host cells.

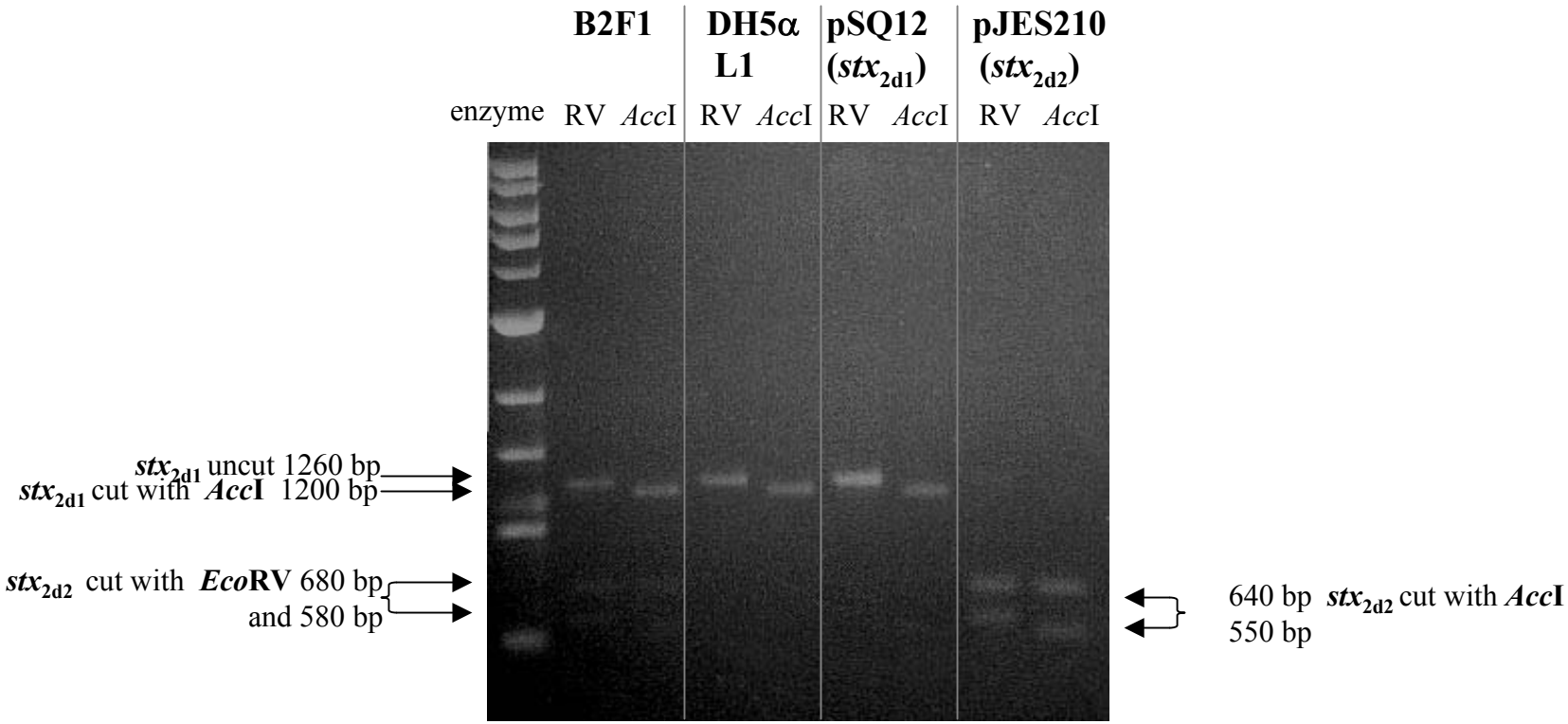


Figure 13. Identification by restriction digest of the Stx2d toxin gene transduced into strain DH5α from strain B2F1. Restriction digests of PCR fragments amplified with primers LT2 and JCS2 from chromosomal DNA of B2F1 ($stx_{2d1}$and stx_{2d2}), putative DH5α lysogen L1, and cosmid clones of stx_{2d1} (pSQ12) and stx_{2d2} (pJES210) are shown. Digested PCR products from putative DH5α lysogen L1 give fragments that coincide in size to those of stx_{2d1}.

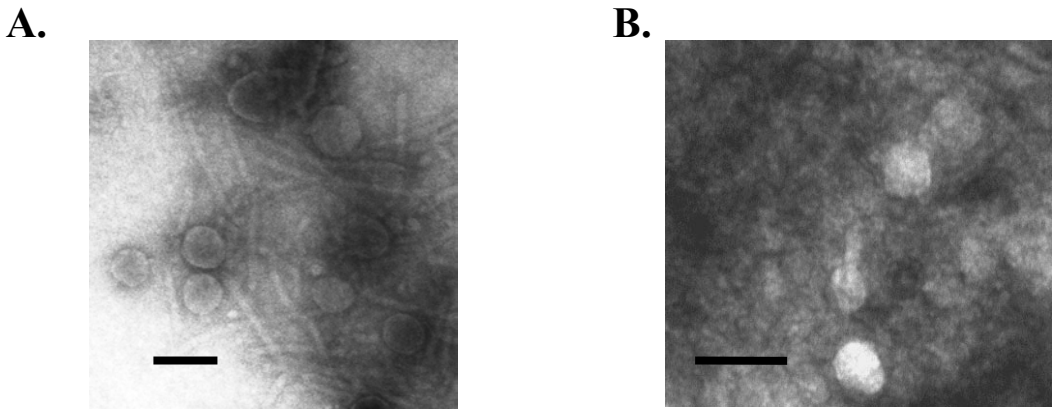


Figure 14. Transmission electron micrograph of ϕB2F1. Panel A.shows a phage preparation from a cell extract of the B2F1 *stx*$_{2d2}$ mutant (7-4) after induction with mitomycin C. Filaments are thought to be flagella from this motile strain. Panel B. shows a phage preparation from a cell extract of DH5α L1,lysogenized with the Stx2d1 toxin-converting phage from B2F1. Bars measure100 nm.

E. Electron microscopic examination of phage from the Stx2d1-producing mutant of strain B2F1 and from the RecA-complemented lysogen of strain DH5α . Bacteriophage particles were observed by transmission electron microscopy from lysates of induced broth cultures of the Stx2d1-producing mutant of B2F1 (Fig. 14A). The stx_{2d1}-converting bacteriophage, designated ϕB2F1, appeared morphologically similar to the stx_2-converting phage, 933W. The head appeared to be a regular hexagonal shape. Filaments were also seen that were quite long and did not appear to be attached to the hexagonal particles. I speculated that the strands were flagella, because this preparation was made from the motile B2F1 toxin mutant that produces Stx2d1 and were absent in a preparation made from the non-motile RecA-complemented lysogen of DH5α (Fig. 14B).

III. Investigation of potential regulators of stx_{2d2} expression

A. Influence of pSQ19 on stx_{2d2} expression *in trans* in strain DH5α. Dr. Susanne Lindgren made the observation that strain DH5α transformed with pSQ545 that contains a 2.8 kb Stx2d2-encoding fragment (*Pst*I-*Eco*RI), was 100-fold more cytotoxic than the same strain transformed with a larger toxin clone containing a 4 kb *Sal*I-*Eco*RI Stx2d2-encoding fragment (pSQ544). She reasoned that the 1.2 kb upstream region, absent in pSQ545, encoded a factor that negatively influenced toxin expression in pSQ544. To test this theory, she subcloned a 1.9 kb fragment from pSQ544 that included the 1.2 kb upstream region missing from pSQ545 and ligated it into pKS^- to make pSQ19. She then co-transformed this recombinant plasmid into DH5α with a compatible toxin gene clone (pSQ547) and measured cytotoxicity of culture lysates. She observed that toxin expression of pSQ547 in DH5α co-transformed with pKS^- was 25- to 40-fold higher than

in DH5α co-transformed with pSQ547 and pSQ19. I repeated this experiment and observed a 21-fold reduction in the cytotoxicity of DH5α transformed with pSQ19 and pSQ547 compared to the cytotoxicity seen with pSQ547 and vector pKS alone. In addition, I tested the cytotoxicity of cell lysates from DH5α co-transformed with pSQ19 and an *stx*$_{2d1}$-bearing plasmid, pSQ347. There was no difference in cytotoxicity between DH5α co-transformed with pSQ347 and pSQ19 compared to DH5α harboring pSQ347 and vector alone. This result suggested that pSQ19 influenced Stx2d2 expression but not Stx2d1 expression.

The pSQ19 insert was sequenced to determine whether a potential regulatory gene was present that might down-regulate Stx2d2 expression (Fig. 15). This pSQ19 sequence revealed for the first time (the toxin gene-flanking regions from the cosmid clones described on pages 52-54 were sequenced subsequently) that the region 500 bp upstream of *stx*$_{2d2}$ was 96% identical to the corresponding region upstream of *stx*$_2$ in phage 933W (Plunkett III *et al.*, 1999). In previous studies from this laboratory, the transcriptional start site of *stx*$_2$ in 933W had been mapped and a putative promoter region between -150 and -123 upstream identified (Sung *et al.*, 1990). I reasoned that the putative *stx*$_{2d2}$ promoter might be located in the corresponding region upstream of *stx*$_{2d2}$ because of the strong homology between these *stx*$_{2d2}$- and *stx*$_2$-flanking regions. Moreover, the region upstream of this putative promoter was homologous to the *ileX* tRNA upstream of *stx*$_2$ in several EHEC strains (Schmidt *et al.*, 1997).

The sequence in pSQ19 further upstream from the tRNA gene was different from the corresponding region in 933W. An 813 bp ORF with partial homology to a

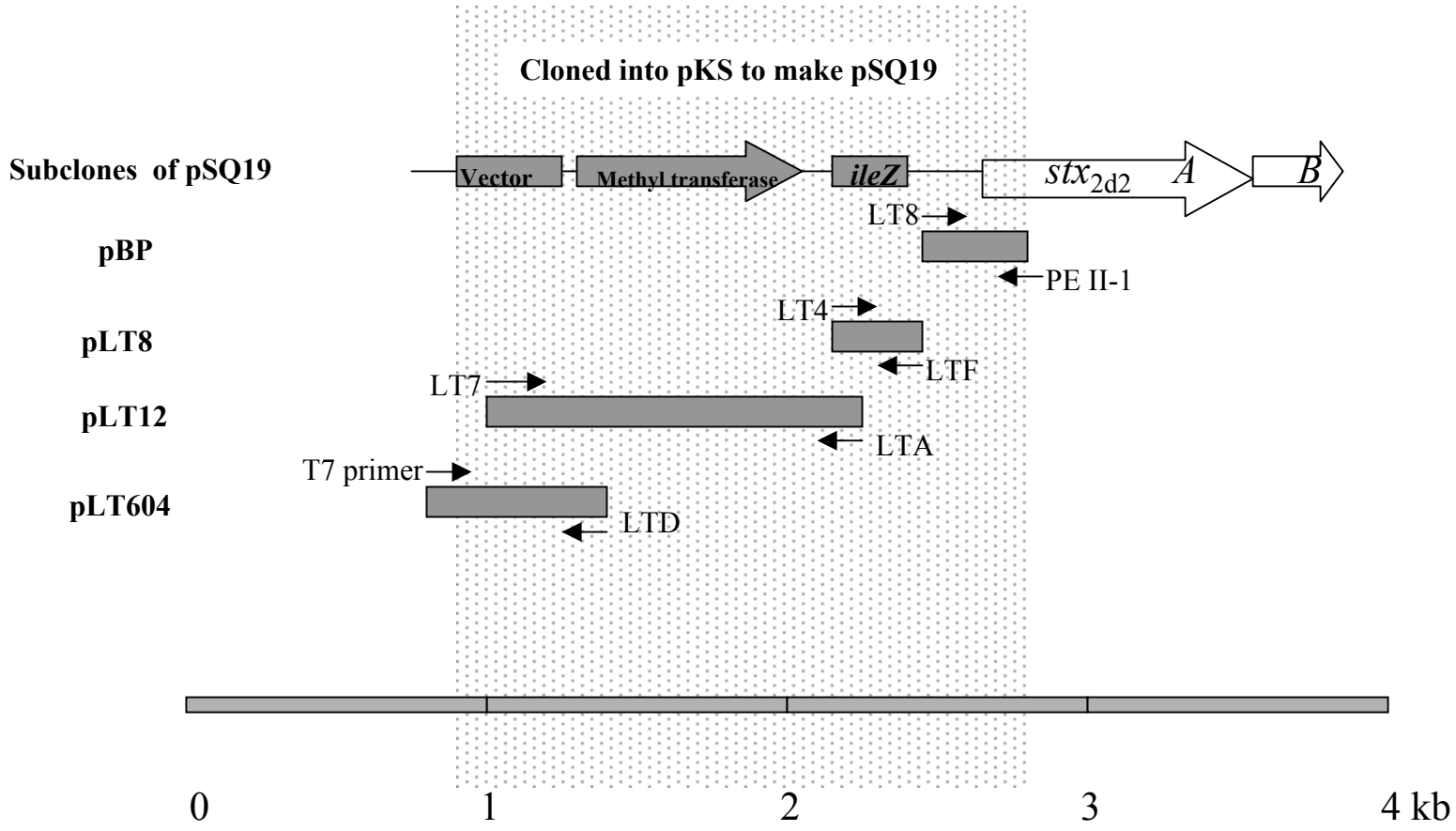


Figure 15. Diagram of the region upstream of *stx*$_{2d2}$. The fragment cloned into pSQ19 is shown in the stippled area and the subclones made from the pSQ19 insert are shown with the PCR primers used to generate each fragment.

bacteriophage methyl transferase was identified on the pSQ19 fragment. Upstream beyond that ORF the distal portion of the insert contained a 460 bp fragment of DNA that was probably of cloning vector origin, as suggested by a BLAST search. The DNA sequence obtained from pSQ19 did not clearly suggest a regulatory element. Only the putative DNA methyl transferase, a DNA modifying enzyme, seemed to be a logical candidate as a repressor of toxin expression. Nevertheless, four fragments (including the cloning vector "scrap" DNA) from the pSQ19 insert were amplified by PCR (Fig. 15) and cloned into the pKS vector to assess the impact, if any, of each DNA segment on toxin expression.

B. Effect of the subclones of pSQ19 on *stx*$_{2d2}$ expression *in trans* in strain DH5α.

Cytotoxicities of cell extracts from DH5α co-transformed with pSQ547 and the individual subclones of pSQ19 were compared with the cytotoxicity of extracts from DH5α co-transformed with pSQ547 and vector alone. The putative promoter region was the only subclone that showed an appreciable reduction of cytotoxicity *in trans* to pSQ547 in DH5α (Fig. 16). The putative promoter clone was associated with a 6.3-fold reduction in cytotoxicity, in contrast to the 22-fold reduction seen with the intact region in pSQ19. I hypothesized that additional copies of the promoter region supplied *in trans* competitively bind a transcriptional activator of toxin expression and reduce its availability and influence on the *stx*$_{2d2}$ clone. I speculated that the putative activator may bind more effectively in the context of the entire pSQ19 fragment than with the promoter region alone, or, that binding may be facilitated by a secondary, weaker, binding point further upstream. Furthermore, I theorized that a transcriptional activator of *stx*$_{2d2}$

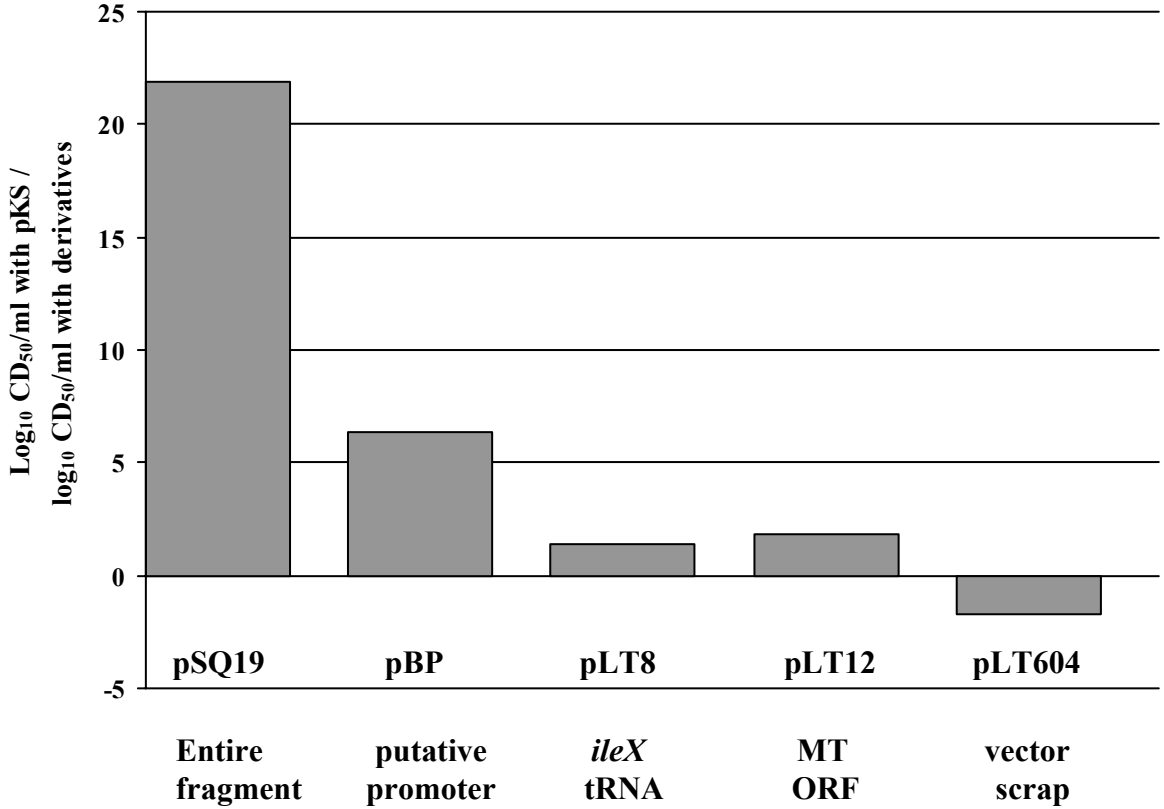


Figure 16. Fold reduction* in cytotoxicity of strain DH5α co-transformed with pSQ547 (stx_{2d2} clone) **and the pSQ19 derivatives versus its cytotoxicity when co-transformed with pSQ547 and pKS vector.**
MT, methyl transferase ORF.

*fold reduction is a ratio of geometric mean values (5 exp.) of $\log_{10}$ CD_{50}/ml with vector alone/$\log_{10}$ CD_{50}/ml with pSQ19 or its derivatives. A negative value results if the denominator is greater than the numerator, which indicates an increase in cytotoxicity, rather than a reduction.

expression in DH5α, which is a non-toxigenic organism, would be a general activator of gene expression and one that would be present in other strains of *E. coli* such as B2F1. The cytotoxicity of strain B2F1 transformed with the putative stx_{2d2} promoter region in a high copy number vector was tested and a ten-fold reduction in cytotoxicity was observed compared to B2F1 transformed with vector alone. (There was no effect on cytotoxicity in B2F1 when transformed with the putative promoter borne on a lower copy number vector.) The proposed effect on toxin expression in DH5α, coupled with the observation of a similar reduction of cytotoxicity in wild-type B2F1 transformed with the promoter region clone, prompted the next series of experiments to identify the putative activator.

C. Transposon mutagenesis of strain DH5α. Mutations were introduced into DH5α by conjugation with *E. coli* strain S17-1λ*pir* transformed with the mobilizable pi-dependent suicide vector, called pATM161. Plasmid pATM161 contains a mini-Tn*5* transposon that encodes a kanamycin resistance gene. This plasmid is classified as a suicide vector because the origin of replication is inactive in the absence of the *pir* gene product. Growth in a pir^- host, such as DH5α, in the presence of kanamycin, forces transpositions of the resistance marker to the host chromosome. I selected DH5α recipients on kanamycin-containing agar (as well as naladixic acid to prevent the growth of the donor strain) to isolate organisms in which the mini-Tn*5* Km^r had inserted randomly into genes on the chromosome. To identify mutations in genes of potential activators of toxin expression, I transformed DH5α with a reporter plasmid, pLTRC, that contained an stx_{2d2}::*lacZ* transcriptional fusion. I selected mutant colonies that did not produce beta-galactosidase, *i.e.* formed white colonies in the presence of X-gal, an indication that the transcription was not initiated at the toxin gene promoter (Fig. 17).

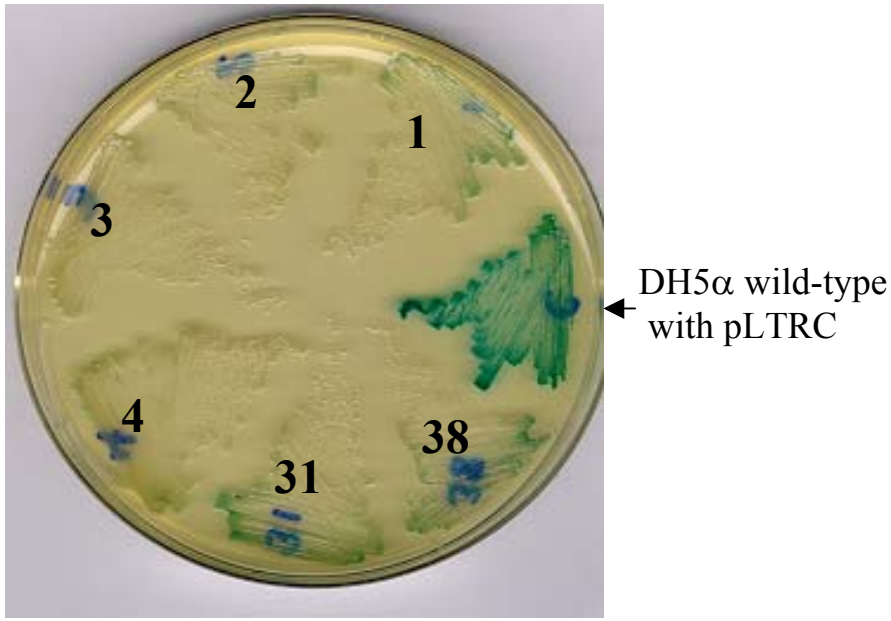


Figure 17. Photograph of strain DH5α mutants and wild-type DH5α transformed with pLTRC and grown on LB agar with X-gal. White phenotype is exhibited by DH5α mini-Tn*5* Km^r mutants transformed with pLTRC in contrast to blue phenotype of wild-type DH5α transformed with pLTRC.

Table 5. Beta-galactosidase assay results conducted on DH5α mini-Tn*5* Km^r insertion mutants that demonstrated a white phenotype on X-gal agar when transformed with pLTRC.

Mutant number	1 hour	2 hours	3 hours
1	4.8	6.2	6.4
2	Not detectable	0.62	1.2
3	Not detectable	1.1	1.2
4	Not detectable	0.3	0.1
7	3.2	6.0	6.5
8	4.6	5.2	6.4
9	3.7	4.2	4.8
16	6.5	7.2	7.5
31	4.1	4.8	5.7
32	3.4	4.4	5.4
72	3.7	5.1	5.1
38	2.6	4.2	4.4
pLTRC in wild-type	20.6	20.5	20.7

Overnight cultures were diluted 1:10 and incubated with ONPG for 1, 2, and 3 hours. Results are expressed as Miller Units.

The efficiency of conjugation was low, 2×10^{-10} to 5×10^{-9}, due to the short mating time (1 to 3 h) necessary to prevent lysogenization of the recipient DH5α with λ-*pir*. Nonetheless, of 3830 colonies screened, 13 white colonies were isolated. Of those, 12 had chromosomal mutations and one had a *lacZ* reporter mutation. Broth cultures of the chromosomal mutants and DH5α transformed with pLTRC were prepared to compare growth kinetics and to assay for beta-galactosidase (Table 5). The mutants and DH5α grew equally well and, the beta-galactosidase assays correlated with the visual phenotype observed. From these results, I concluded that the white phenotype represented low beta-galactosidase activity and not a failure of the mutants to grow adequately.

Chromosomal DNA samples from the 12 mutants were digested with restriction enzymes and evaluated by Southern analysis with the kanamycin resistance gene as a probe to locate the restriction fragments into which the transposon had inserted (Fig. 18). Six of the 12 mutants contained more than one chromosomal insertion and were not evaluated further. The concentration of kanamycin used for selection of transposon mutations was 50 μg/ml. DeLorenzo *et al.* (1990) used kanamycin concentrations between 25 and 75 μg/ml to select for mini-Tn*5* Km^r mutations in *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* is inherently more resistant to aminoglycosides than members of the *Enterobacteriaceae*, therefore it is possible that50 μg/ml kanamycin favored multiple insertions in *E. coli.* There were six individual insertion mutations, designated mutants 1, 2, 3, 4, 31, and 38. Southern blots made from four different restriction enzyme digests of chromosomal DNA showed that mutants 1 and 4, and mutants 2 and 3, consistently appeared on the same size fragments and were regarded as

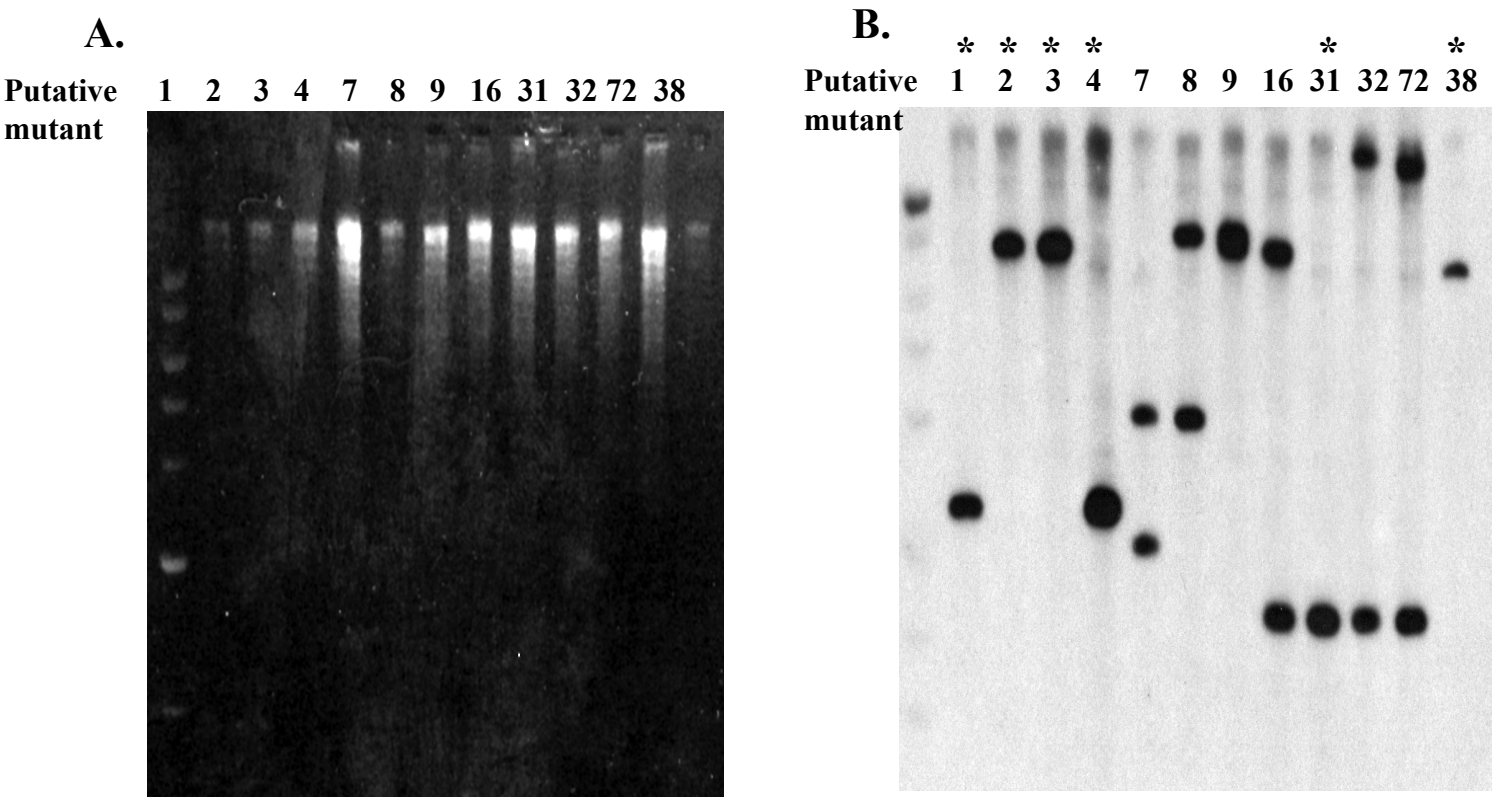


Figure 18. Identification of strain DH5α DNA restriction fragments that contain mini-Tn*5* Km^r insertions. A. *Sac*II digest of chromosomal DNA extracted from 12 putative activator mutants of DH5α that gave white colonies on LB agar in the presence of X-gal.
B. Southern blot of the digest shown in A. probed with the kanamycin resistance gene from the mini-Tn*5* Km ^{r}. Mutants with single transposon insertions are marked with (*).

probable siblings since they were harvested from the same mating mixture. Mutations 1 and 4 were later confirmed by sequencing to be inserted into identical sites.

1. Influence of DH5α transposon mutations on toxin production. The mutants of DH5α had been selected based on the white phenotype displayed when transformed with the *stx*$_{2d2}$::*lacZ* promoter fusion (Fig. 17). The next step was to determine whether the expression of Stx2d2 was impaired in these mutants as well. DH5α mutants 2, 4, 31, and 38 were cured of the reporter plasmid and transformed with pSQ545, an *stx*$_{2d2}$ clone in pKS$^-$ (oriented in the opposite direction of the plasmid-encoded *lacZ* promoter), pSQ547, (an *stx*$_{2d2}$ clone in the medium copy number vector pACYC184), or pSQ343, (an *stx*$_{2d1}$ clone in pKS$^-$in the opposite orientation from the vector *lacZ* promoter). A clone of *stx*$_2$ in the pSK vector was transformed into the mutants and wild-type as well. Cytotoxicities of culture lysates from mutants 2, 4, 31, and 38 transformed with toxin clones were compared with that of lysates from DH5α transformed with the same toxin-encoding plasmids. Culture lysates from all of the mutants were less cytotoxic than similarly transformed wild-type DH5α lysates. The mutants transformed with the *stx*$_{2d2}$ gene clone (pSQ547) in a medium copy number vector (pACYC184) showed only a 4 to 5 fold reduction in cytotoxicity compared to wild-type (Table 6). However, the reduction observed was reproducible and within each assay the wild-type was always more toxic than mutants 2, 4, 31 or 38. Ratios were calculated based on CD_{50}/CFU to take into account minor fluctuations in culture densities.

The differences in cytotoxicity of cell lysates from the DH5α mutants and wild-type DH5α were profound when they were transformed with pSQ545, a high copy

Table 6. Comparison of cytotoxicity of culture lysates of DH5α and the mutants of strain DH5α transformed with pSQ547 (*stx*$_{2d2}$ in pACYC184).

Mutant #	Fold reduction in cytotoxicity of mutants compared to wild-type similarly transformed* Represents the results of 3 - 4 assays
2	4.2
4	4.2
31	5.4
38	5.5

* Reduction was calculated as the ratio of $\log_{10}$ of geometric means of CD_{50} per $\log_{10}$ CFU of wild-type/ $\log_{10}$ CD_{50} per $\log_{10}$ CFU mutant.

number vector clone of *stx*$_{2d2}$ (Table 7). Cytotoxicity of cell lysates from the mutants was reduced two to three logs when compared to the DH5α wild-type lysates. From comparison of the cytotoxicity results obtained with low and high copy number toxin-encoding plasmids, it appeared that the greater the amount of toxin expressed from a clone, the more dramatic the observable reduction in cytotoxicity of the mutants. None of the mutants appeared to exert a significantly greater influence on toxin expression than the others. In addition, I transformed the DH5α mutants and wild-type with clones of *stx*$_2$ and *stx*$_{2d1}$ in high copy number vectors. Expression of both toxins was dramatically reduced in the mutants (Table 7). Colony counts were determined on the cultures used for cytotoxicity assays. The mutants grew as well as strain DH5α transformed with pSQ547, but wild-type DH5α transformed with toxin clones in high copy number vectors yielded colony counts one to two logs lower than the mutants. The observation that expression of Shiga toxins at high levels is detrimental to bacteria has been made repeatedly in our laboratory (unpublished observation). Optical densities of the cultures were comparable, despite the drop in colony count seen in the high level toxin-producers. I reasoned that the cultures had probably grown to comparable densities but viability of the high toxin-producers declined more rapidly. Therefore, rather than standardize the CD_{50} by CFU for transformants with high copy number toxin vectors, I compared cytotoxicities per ml of cultures with comparable optical densities.

Vero cells are exquisitely sensitive to the Shiga toxins. Thus, I considered that the sensitivity of the assay might exaggerate the actual difference in concentration of toxin expressed. Therefore, I compared the amounts of toxin antigen produced by DH5α with the levels produced by the mutants (with the assistance of Edda Twiddy). Dot blots were

Table 7. Reductions in cytotoxicity of culture lysates from strain DH5α and the mutant of DH5α transformed with various high copy number toxin clones. Cultures were standardized by optical density. Values given are the log reduction ratios, i.e. calculated from geometric means of $\log_{10}$ CD_{50} per ml DH5α wild-type/Log_{10} CD_{50} per ml mutant.

Strain	pSQ545* (*stx*$_{2d2}$ in pKS)	pSQ343** (*stx*$_{2d1}$ in pKS)	pMJSK** (*stx*$_2$ in pSK)
Mutant 2	2.51	2.25	2.15
Mutant 4	2.35	2.07	2.39
Mutant 31	2.44	2.78	2.28
Mutant 38	2.7	3.00	2.28

*Results of three to four experiments **Results of one experiment

prepared from the same cell lysates of pSQ545 transformants used for cytotoxicity assays. The blots were probed with anti-Stx2 monoclonal antibody, and the results were compared to known concentrations of Stx2 (Figure 19). The concentrations of protein expressed from mutants and wild-type showed the same logarithmic difference observed in Vero cell assays. Therefore, I concluded that the cytotoxicity data represented the actual toxin protein expressed in the mutant and wild-type cultures.

Another concern in evaluation of toxin expression in the mutants versus wild-type was that the mutations might exert a general effect on plasmid copy number unrelated to toxin expression. To address this concern, I extracted plasmid DNA from 1.5 ml broth cultures of the Tn*5* mutants and wild-type DH5α that were transformed with pSQ545. I loaded equal volumes of the plasmid extracts onto agarose gels and visually compared the amount of plasmid DNA in these extracts (Fig. 20). Colony counts were obtained on the same cultures. There appeared to be about twice as much plasmid DNA isolated from stx_2 and stx_{2d1} in high copy number vectors. Expression of both toxins was dramatically reduced in the mutants (Table 7). Colony counts were determined on the cultures used for cytotoxicity assays. The mutants grew as well as DH5α transformed with pSQ547, but wild-type DH5α transformed with toxin clones in high copy number vectors yielded colony counts one to two logs lower than the mutants. The observation that expression of Shiga toxins at high levels is detrimental to bacteria has been made repeatedly in our wild-type DH5α compared to the mutants, but not a great enough difference to suggest a two to three log difference in toxin expression.

Another approach to assessing plasmid copy number was to compare beta-galactosidase levels in mutants and wild-type transformed with pBC and pKS vectors

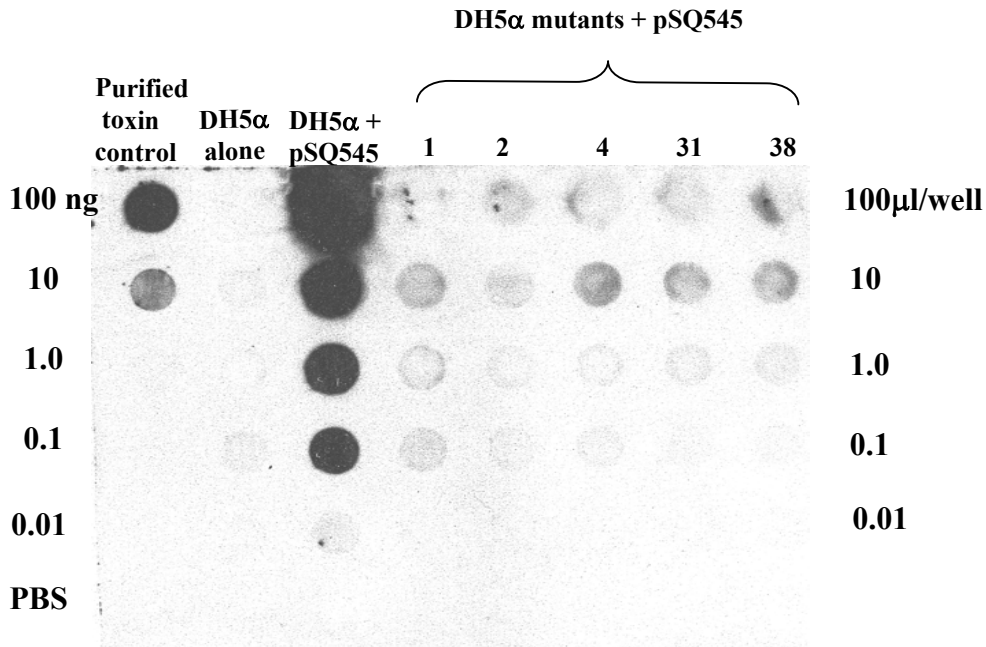


Figure 19. Dot blot comparison of Stx2d2 toxin production in DH5α and the DH5α mutants. The filter was prepared with sonicated extracts of DH5α and DH5α mutants transformed with pSQ545 that encodes $stx_{2d2.}$ and it was probed with monoclonal antibody 11E10 against the A subunit of Stx2 (Perera, *et al.*, 1988).

Mutant	CFU/ml culture
1	2.8×10^8
2	4.3×10^8
3	5.5×10^8
4	1.8×10^8
7	1.1×10^8
8	1.9×10^8
9	1.5×10^8
16	1.2×10^8
31	9.0×10^6
32	2.2×10^8
72	9.4×10^7
38	2.7×10^8
DH5α	2.3×10^7

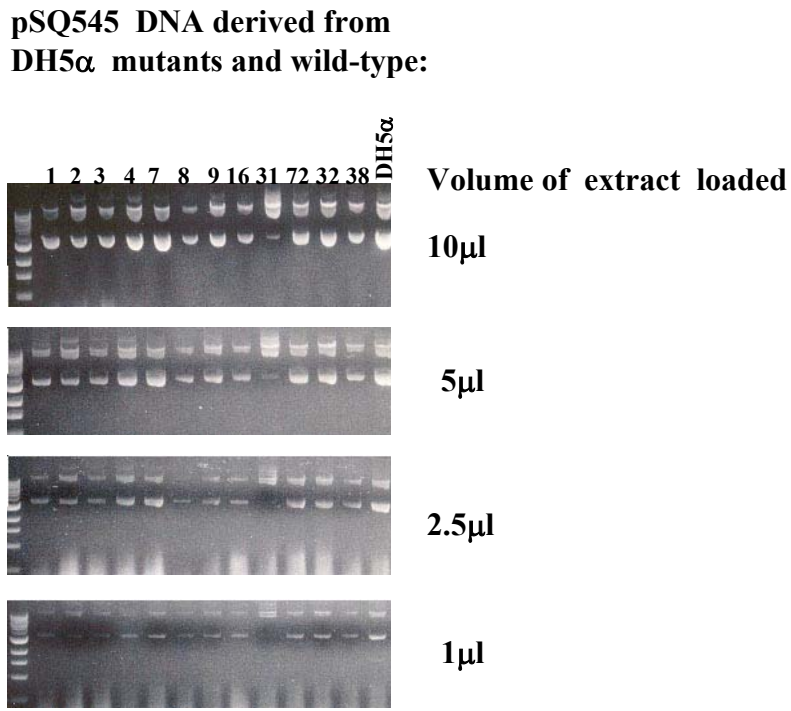


Figure 20. Comparison of the amounts of pSQ545 plasmid DNA extracted from cultures of DH5α and the DH5α transposon mutants. Alkaline lysis preparations were made from 1.5 ml cultures of each mutant at the same time. Colony counts for these cultures appear in the table on the left. Growth of mutant 31 in this experiment was considerably less than the typical 10^8- 10^9 CFU/ml. For most of the mutants about half as much plasmid DNA was obtained from each plasmid preparation as from wild-type DH5α.

alone (the former was the reporter vector, and the latter the toxin gene vector). The mutants expressed less beta-galalactosidase than wild-type (Table 8). However, this difference was not even two-fold (the visually estimated difference in concentration of plasmid DNA shown in Fig. 20) and, therefore, not of the same order of magnitude as demonstrated for the reduction in toxin expression in the mutants compared to the wild-type DH5α. Additionally, the expression of an unrelated *E.* gene, *tir*, that had been cloned into pKS, was compared in the Tn*5* mutants and wild-type DH5α. Standardized cell extracts were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for Western blot analysis with anti-Tir antibody. We observed the opposite effect on *tir* expression from that seen with β-galactosidase. In contrast to the toxin genes, the Tir gene was expressed to a somewhat greater extent in the mutants that in wild-type DH5α. From these findings, I concluded that the DH5α mutants displayed dramatically reduced toxin expression compared to wild-type as observed both by cytotoxicity for Vero cells and by toxin protein concentration on dot blot. In addition, these differences could not be attributed solely to variation in plasmid copy number and was specific for toxin expression.

Finally, an effort was made to test the influence of these mutations on expression of a single chromosomally encoded toxin gene. I had observed reduced expression of acloned stx_2 gene in the DH5α mutants compared to wild-type. Therefore, with the help of Clare Schmitt, I lysogenized the mutants and DH5α with the stx_2-encoding phage 933W and tested the cytotoxicity of the lysogens. None of the lysates from the lysogens including those of DH5α wild-type, were cytotoxic. We had previously observed a rather high level of toxin expression without induction of 933W in strain C600 (Fig. 9) and had

Table 8. Fold reduction in beta-galactosidase production* for the DH5α mutants transformed with two different *lacZ*-encoding vectors compared to wild-type DH5α similarly transformed.

Mutant number	pBC	pKS
2	1.3	1.6
4	1.2	1.6
31	1.2	1.7
38	1.2	1.7

*Beta-galactosidase assay performed on transformed strains without induction.

hoped that there would be enough expression of Stx2 in the DH5α background to assess the influence of the mutations on expression of the *stx*$_2$. However, it appeared that phage repression in the absence of a functional RecA was essentially complete, as reported by others (Fuchs *et al.*, 1999), and no conclusions could be drawn regarding the influence of a potential activator of toxin expression on toxin expression from the 933W lysogens. [As reported earlier, I obtained a *recA* clone that I used to complement the DH5α(ϕB2F1) lysogen; however that clone was not available at the time these lysogens were made.]

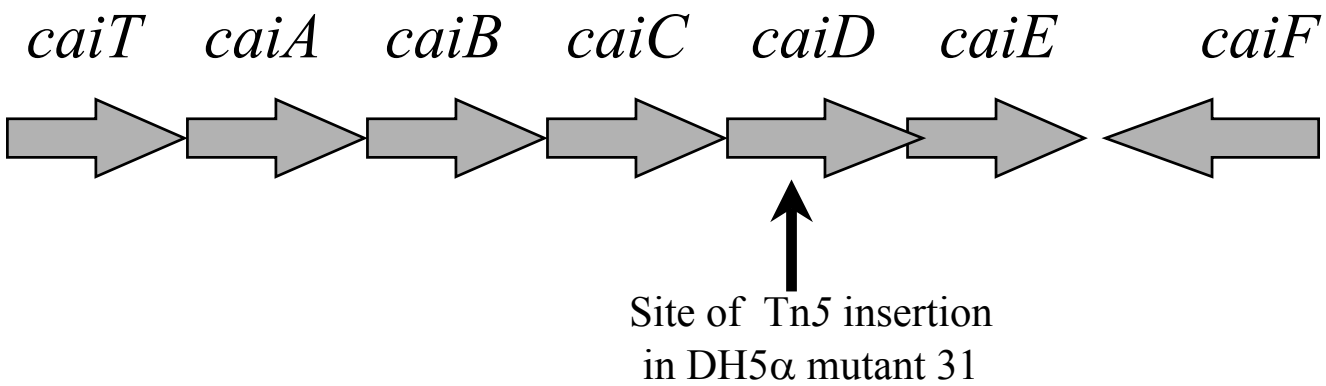
2. Identification of the mini-Tn*5* Km^r insertion sites in DH5α. The next step was to determine the nature of the mutations in DH5α. The mutations were cloned from mutants 1, 4, 31 and 38 by isolating fragments of the expected size ranges (previously determined by Southern blot) from digests of chromosomal DNA and ligating them into pKS. Transformants that carried the correct insertions were readily selected by growth on kanamycin agar. Despite repeated efforts with digests generated from two different enzymes, I was unable to isolate a clone of the mutation from mutant 2 or mutant 3. The other interrupted genes were identified by sequencing outward from the transposon into the flanking sequences that were then compared against the K-12 genome in GenBank.

Mutants 1 and 4 contained identical insertions and were probably siblings. The mini-Tn*5* insertion site in those mutants was in the DH5α homologue of a gene designated b2321 in K-12. This gene is a putative cell division factor also referred to as "div" by Blattner *et al.* (Blattner *et al.*, 1997). There is another homologue of this gene on the MG1655 chromosome. *E. coli* O157:H7 933EDL strain also contains the two "div"

homologues (Perna *et al.*, 2001). Thus a mutation in one copy of "div" is unlikely to be lethal to the cell. That the insertions in mutants 1 and 4 may have exerted a polar effect on the downstream gene *pdxB,* (encodes an enzyme that mediates steps in the biosynthesis of the essential coenzyme pyridoxal 5'-phosphate) could not be ruled out. In addition, a somewhat less homologous region described as an unknown prophage-encoded protein is associated with cryptic bacteriophage CP-933T in the EDL933 strain. Mutant 31 had an insertion in the DH5α homologue of the *caiD* gene (Fig. 21). *caiD* encodes an enzyme of the carnitine metabolic pathway described as a D-, L- carnitine racemase and a crotonobetainyl-CoA hydratase that serves to convert D-carnitine to L-carnitine (Eichler *et al.*, 1994) as well as to convert crotonobetaine to L-carnitine (Elssner *et al.*, 2001). L-carnitine is the form in which carnitine is used in the bacterial cell (Fig. 22). The role this gene might have as a global regulator is unclear; however, the *caiD*-coding region contains the promoter for *caiE*, a gene that is proposed to be an activator for the *cai* operon (Eichler *et al.*, 1994) and potentially others (Fig. 21).

Mutation 38 was located within a gene designated as *ycdU*, or b1029, in *E. coli* K-12. The *ycdU* allele encodes a hypothetical 328 amino acid protein with 8 possible transmembrane regions. No function or homologue for this gene has been identified (Blattner *et al.*, 1997).

In sum, none of the mutational insertions in DH5α occurred in genes that readily suggested a role in regulation. However, the existence of homologous genes in B2F1 was verified by PCR amplification of the corresponding genes with primers derived from the



caiT product = carnitine transporter protein
caiA product = carnitine dehydratase
caiB product = crotonobetaine reductase
caiC product = acetyl-CoA ligase
caiD product = carnitine L- D- racemase
caiE product = possible co-activator of *cai* and *fix* operons with *cai F* product
caiF product = transcriptional activator of *cai* and *fix* operons

Figure 21. Genetic arrangement of the carnitine operon of *Escherichia coli* K-12 strain MG1655

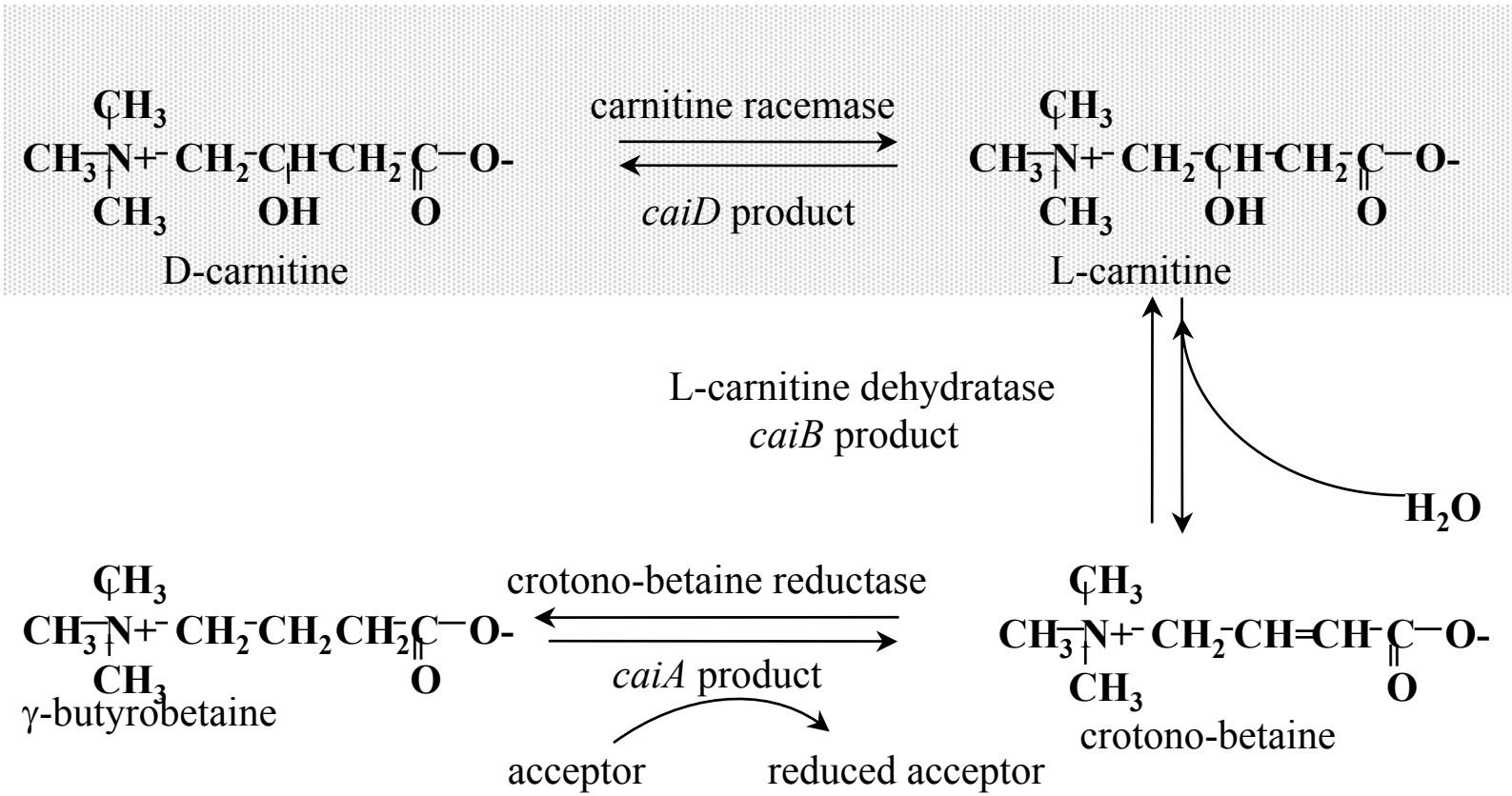


Figure 22. **Carnitine metabolism in *Escherichia coli.*** The reaction highlighted with the stippled background is catalyzed by the *caiD* gene mutated in strain DH5α mutant 31.

K-12 sequences. The PCR products from B2F1 were cloned and sequenced and showed 97% or greater identity to their K-12 counterparts.

3. Complementation of the DH5α mutations. Complementation of the reduced toxin expression phenotype in the DH5α mutants was difficult. At least five different complementing clones were needed to test the interrupted genes and potential polar mutations in DH5α. Unique restriction sites into which the wild-type genes could be ligated were limited in the low copy number vector pWSK130; that vector had already been engineered to contain two antibiotic resistance markers. In addition, there were no antibodies available to identify the gene products of the complementing clones, and a biochemical assay was available for only one of the proteins encoded by a complementary gene (*caiD*). Therefore, I was unable to determine if the complementing clones expressed the desired proteins. Nonetheless, I transformed the clones that I was able to construct, *caiD*, *caiE*, *caiDE* and *ycdU*, into DH5α and the corresponding mutants of DH5α also transformed with the Stx2d2 toxin gene clone pSQ545. I did not observe any restoration of cytotoxicity to DH5α or the mutants in the presence of the cloned wild-type genes. The presence of *caiE in trans* to the toxin gene clone in DH5α and mutant 31 resulted in ablation of cytotoxicity altogether. Re-transformation of the toxin plasmid (pSQ545) DNA from those two hosts into a fresh culture of DH5α revealed that pSQ545 was no longer toxigenic. Although it appeared that *caiE in trans* to pSQ545 might have promoted the acquisition of some mutation in the toxin clone, the same phenomenon was not seen in the presence of the *caiDE* clone. Thus, no conclusions could be drawn from these observations. Due to the problems described and the lack of reagents needed to

assess gene expression, the complementation studies were abandoned to focus on the influence of the mutations in B2F1 (subsection after next).

4. *In vivo* studies with the strain DH5α mutants. To determine if the reduced toxin expression seen *in vitro* with the DH5α "div", *caiD*, and *ycdU* mutants transformed with stx_{2d2} also occurred *in vivo,* I isolated spontaneous streptomycin-resistant mutants of each and transformed them with pSQ545. Mice were treated with 5 g/L of streptomycin and 5 g/L of ampicillin, the resistance marker encoded in pSQ545. Six mice were treated with antibiotics and fed no bacteria. I fed 10^{10} CFU of various DH5α strains to groups of 6 mice as follows: i) DH5α wild-type transformed with pSQ545; ii) DH5α wild-type transformed with pKS, and; iii) DH5α mutants 4, 31, and 38 transformed with pSQ545. The heavy inoculum fed to these mice was necessary to achieve colonization since DH5α apparently lacks some of the factors necessary for establishment of infection (Sung *et al.*, 1990). I verified that the mice were colonized by fecal cultures; however, the levels of colonization were substantially lower than the levels usually observed in the streptomycin mouse model with pathogens such as B2F1 (Str^r). Only between 10^2 and 10^4 CFU /g feces were evident two days after the DH5α strains were fed to streptomycin-treated mice. Conversely, B2F1 (Str^r)-infected mice shed 10^8 CFU/g feces even when fed considerably fewer organisms (as low as 10^3CFU).

Despite the difficulty in establishing infection in mice fed DH5α, three of the six mice fed wild-type DH5α transformed with pSQ545 died with a mean time to death of 4.3 days (Table 9). However, none of the mice given the *caiD* mutant died. Additionally, two of the six fed the *ycdU* mutant died but the mean time to death was 8.5

days. Lastly, two of the six mice fed the "div" mutant died with a mean time to death of 13.5 days. As expected, none of the uninfected, antibiotic-treated mice died, and none of the DH5α and vector control mice died. I concluded from this preliminary experiment (to be repeated by a technician in our laboratory) that the reduced toxin expression seen *in vitro* with the DH5α mutants transformed with pSQ545 translated into extended the mean times to death for the animals fed the *ycdU* and "div" gene mutants and reduced virulence of the *caiD* mutant *in vivo*.

D. Characteristics of the insertion mutations introduced into B2F1 by allelic exchange. The most important issue to address next was whether the DH5α mutations that resulted in reduced expression of plasmid-encoded toxin genes would result in reduced levels of toxin production in B2F1 from only one chromosomal copy of stx_{2d2}. I had established that B2F1 contained homologous genes to "div", *caiD*, and *ycdU* by PCR amplification. The corresponding B2F1 genes were highly similar to their DH5α counterparts, so the homologous recombination necessary to achieve insertions of the mutated genes from DH5α by allelic exchange was effective. I was able to demonstrate co-integration of pLT14, pLT31 and pLT38, the pSTAMP-based suicide vectors that corresponded to the "div", *caiD*, and *ycdU* mutations in DH5α, into the B2F1 chromosome. These co-integrate strains could be subcultured repeatedly on ampicillin LB agar at 44^{o}C, observations that provided suggestive evidence that the ampicillin resistance gene in each strain was chromosomally located. Subsequent rounds of growth of these strains in the presence of kanamycin, without ampicillin selection, led to the resolution of mutations 4 and 31 ("div" and *caiD*). The presence of a kanamycin

Table 9. Virulence of DH5α and the transposon mutants of DH5α transformed with pSQ545 (*stx*$_{2d2}$ in pKS) in the streptomycin-treated mouse model of STEC infection*.

Bacterial strain fed (dosage 10^{10} CFU)	Deaths/group of mice	Mean time to death (days)
DH5α + pSQ545	3/6	4.3
Mutant 4 + pSQ545 ("div" gene mutant)	2/6	13.5
Mutant 31 + pSQ545 (*caiD* mutant)	0/6	
Mutant 38 + pSQ545 (*ycdU* mutant)	2/6	8.5
DH5α + pKS (vector control)	0/6	
No bacteria fed (antibiotic treatment controls)	0/6	

*Mice were treated with both streptomycin at 5g/L and ampicillin 5g/L.

resistance gene was determined by PCR or colony blot (to distinguish spontaneous kanamycin mutations from actual gene insertions). The insertions of the desired mutations were confirmed with Southern blots probed with the DH5α *caiD* gene or "div"gene to demonstrate acquisition of the 2.4 kb mini-Tn*5* Km^r insertion (Fig. 23 A, B). Despite numerous attempts, I was unable to obtain a resolved *ycdU* mutant. I concluded that, unlike the corresponding mutation in DH5α, the *ycdU* mutant in B2F1 was probably lethal.

The B2F1 *caiD* and "div" gene mutants were tested for cytotoxicity to Vero cells. Contrary to the reduction in cytotoxicity observed with these gene mutations in DH5α transformed with pSQ545, these mutations in B2F1 did not result in reduced cytotoxicity for Vero cells (Fig. 24). Nevertheless, the mutants and B2F1 were fed to mice in doses that ranged up to 10^8 CFU per animal so as to determine the 50% lethal dose of the mutants with that of the parent strain. The death rate in each group and the mean time to death was measured (Table 10). No dose-related response was seen in mice fed B2F1 or the *caiD* mutant. There were fewer deaths in the low dose groups of mice fed the "div"mutant, and an extended mean time to death with the lowest inoculum, suggestive of a dose response. However, at the higher doses the "div" gene mutant killed mice more uniformly than did B2F1 or its *caiD* mutant. Thus, I could not determine whether these lethal dose observations were random or dose-related. The lack of a dose- related response prevented an accurate calculation of LD_{50}s for these organisms and suggested that the smallest inocula given were close to or exceeded the actual LD_{50} for each organism. Overall the death rates and the mean times to death did not differ substantially among the mutants and wild-type B2F1. Thus, contrary to our expectations based on our

observations with mutations in DH5α, the corresponding chromosomal mutations in B2F1 did not display reduced cytotoxicity *in vitro* or reduced virulence in the streptomycin-treated mouse model.

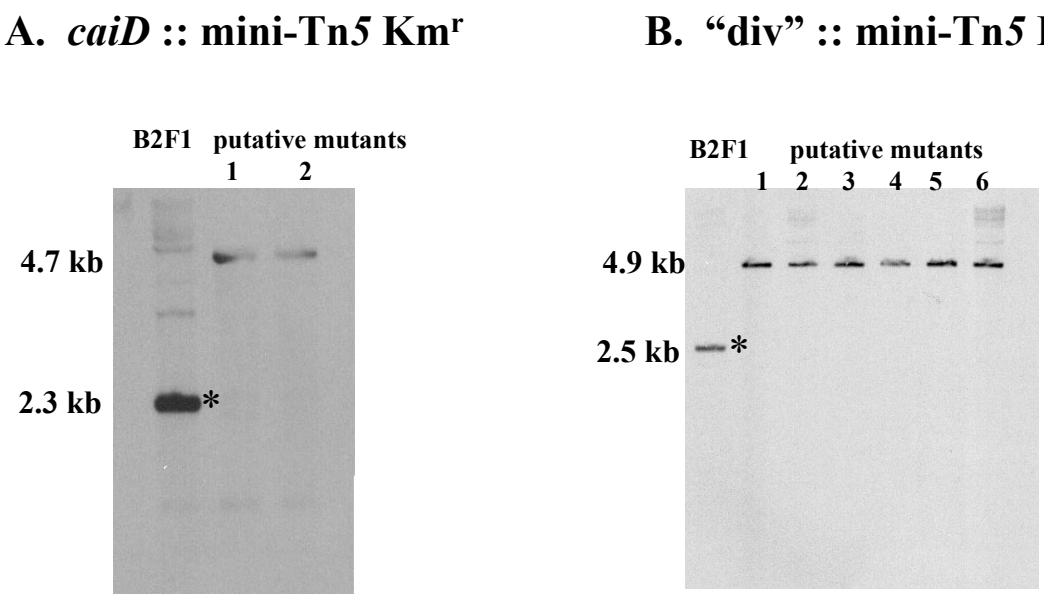


Figure 23. Southern blots of *Eco*RV-digested chromosomal DNA from strain B2F1 and strain B2F1 mutants. **Panel A**. Digest probed with *caiD* gene of DH5α. **Panel B**. Digest probed with "div" gene of DH5α. The native *caiD* and "div" homologues of B2F1 are indicated (*). The higher molecular weight bands represent the 2.4 kb insertion of the mini-Tn*5* Km^r acquired by allelic exchange from DH5α by two to six isolates of each mutant.

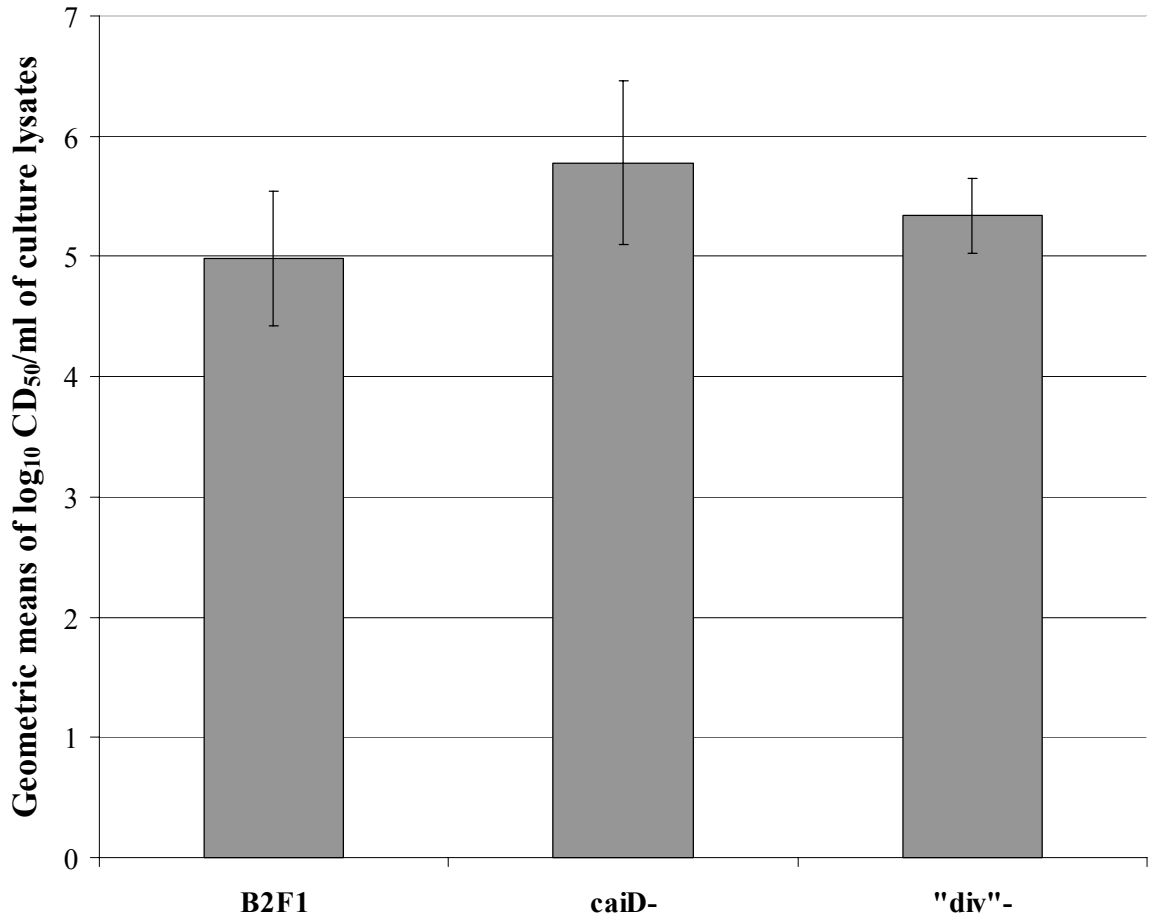


Figure 24. Cytotoxicity to Vero cells of culture extracts from strain B2F1 and the *caiD* and "div" gene mutants of strain B2F1. The B2F1 mutations were introduced by allelic exchange from the corresponding transposon insertion mutations of DH5α. Columns represent $\log_{10}$ geometric means of 5 or 6 assays, and the error bars indicate 95% confidence intervals about the means.

Table 10. Virulence in the streptomycin-treated mouse model of STEC infection of B2F1 and the mutants B2F1 with transposon insertions in *caiD* or "div" genes. (7 groups of 5 mice each/ strain)

Dosage of bacteria fed	B2F1 wild-type		*caiD* mutant		"div" gene mutant	
	deaths/group	TTD* (days)	deaths/group	TTD (days)	deaths/group	TTD (days)
10^2	5/5	5.8	5/5	7.0	2/5	9
10^3	2/5	6.5	5/5	6.6	2/5	5.5
10^4	5/5	6.0	5/5	5.8	5/5	6.4
10^5	3/5	6.6	3/5	5.6	5/5	7.0
10^6	5/5	5.8	5/5	5.6	5/5	5.6
10^7	3/5	6.0	5/5	6.6	5/5	5.2
10^8	5/5	6.0	4/5	6.0	5/5	5.4
cumulative	28/35	6.1	32/35	6.1	29/35	6.3

* TTD, mean time to death

DISCUSSION

As stated in the Introduction, the objective of this research plan was to identify mechanisms of Stx2d regulation in *Escherichia coli* O91:H21 strain B2F1. To accomplish this objective, three specific aims were identified and are reiterated briefly here. The first aim was to compare the expression of Stx2d1and Stx2d2 individually with single toxin mutants of B2F1 and to determine the extent to which each toxin contributes to the toxicity of B2F1 *in vitro* and *in vivo.* The second aim was to determine if there was a toxin-converting bacteriophage in B2F1 and determine the influence of bacteriophage induction on Stx2d expression in the individual toxin-producing mutants. The third aim was to identify the gene responsible for a putative activator of Stx2d2 expression in DH5α and determine the effect on cytotoxicity and virulence in the streptomycin-treated mouse model of mutation(s) in the homologous gene(s) in B2F1.

I. Roles of the two Stx2d alleles in B2F1 toxicity.

B2F1 encodes two virtually identical, functional Stx2d toxins. We have observed in our laboratory, and others have reported (Skinner and Jackson, 1998; Suh *et al.*, 1998), that expression of high levels of Shiga toxin is detrimental to bacterial cell growth. Therefore, we postulated that Stx2d expression in B2F1, like Stx expression in other STEC, is regulated. As a first step in the study of regulation of toxin expression in B2F1, an stx_{2d1}::*cat* knock-out mutant capable of Stx2d2 production was constructed. The corresponding mutation in stx_{2d2} was constructed by Angela Melton-Celsa. I compared the *in vitro* levels of toxin expression in each of the mutants and determined the relative contribution of each toxin to virulence in mice. I observed that the cytotoxicity for Vero

cells of the Stx2d2-producing mutant was equivalent to that of B2F1, and the Stx2d1-producing mutant yielded only about one ninth the level of cytotoxicity attributable to Stx2d2. In the streptomycin-treated mouse model, this difference in toxicity between the Stx2d1- and Stx2d2-producing mutants was crucial to virulence. The Stx2d2-producing mutant was still highly virulent to mice, while the Stx2d1 producer was almost completely attenuated. I concluded that Stx2d2 was primarily responsible for the cytotoxicity and virulence in mice of B2F1. However, it was impossible to determine from these experiments whether Stx2d1 expression was down-regulated, or Stx2d2 expression was up-regulated or both.

II. Effects of bacteriophage induction on Stx2d expression.

Although toxin-converting phage had not previously been isolated from B2F1, preliminary DNA sequencing of the regions upstream of stx_{2d1} and stx_{2d2} revealed that the DNA sequences upstream of each toxin gene shared strong homology to the 500 bp region directly upstream of stx_2 in the inducible bacteriophage 933W. In addition, mitomycin C treatment of STEC strains with chromosomally-encoded toxins, Stx2c and Stx2e, was reported to enhance toxin expression, even in the absence of inducible bacteriophages (Yee *et al.*, 1993; Fujii *et al.*, 1994). Therefore, I decided to test cytotoxicity of the Stx2d toxin mutants after mitomycin C induction and to repeat attempts to isolate a toxin-converting phage from B2F1.

Four lines of evidence derived from this investigation indicated that the stx_{2d1} gene is borne on an inducible toxin-converting bacteriophage in *E. coli* strain B2F1. First, the Stx2d1 toxin gene was transferred to DH5α via a protocol used for transduction, and

the resulting lysogen was cytotoxic when *recA* was supplied *in trans*. Second, Stx2d1 expression was increased *in vitro* and *in vivo* under conditions that are known to induce bacteriophages. Although the *in vivo* induction of elevated toxin expression in the presence of ciprofloxacin therapy was not as pronounced as that which I observed *in vitro*, the biological variations in colonization levels and antibiotic uptake in the mouse model make it difficult to optimize the inductive effect of ciprofloxacin. Nonetheless, the normally attenuated mutant of B2F1 that produces only Stx2d1 became more virulent in the presence of ciprofloxacin. Third, the RecA-dependent nature of Stx2d1 expression in DH5α suggested that Stx2d1 is co-regulated with the bacteriophage late genes involved in the lytic cycle. The low level of expression of Stx2d1 without induction implies that ϕB2F1 is not readily induced spontaneously *in vitro* or in mice. Fourth, electron microscopic examination of lysates from the Stx2d1-producing mutant of B2F1 or the RecA-complemented lysogen of DH5α revealed a phage with morphology similar to the stx_2 -converting bacteriophage 933W.

Comparison of the stx_{2d1}-flanking regions with the GenBank Database revealed genetic as well as structural similarities between the ϕB2F1 and 933W. However, unlike the Stx2-converting phages that have a Q gene approximately 1.2 kb upstream of the toxin genes, ϕB2F1 had no *Q* homologue in the corresponding region upstream of stx_{2d1}. The antiterminator Q is critical for the transcription of stx_1 and stx_2 (Neely and Friedman, 1998; Wagner *et al.*, 2001), and it is puzzling that an intact *Q* homologue was not found in the stx_{2d1} phage. One possible explanation is that there is a *Q* gene homologue encoded further upstream beyond the 4 kb that was sequenced. In fact, I did identify a 50 bp region that is 90% identical to a portion of the 933W *Q* gene roughly 3 kb upstream of

the start of the *stx*$_{2d1}$ gene. However, I was unable to amplify a Q gene product from B2F1 chromosomal DNA with PCR primers specific for the complete 933W Q gene. This finding suggests that a 933W-like *Q* gene was present in that location at some time in the evolution of ϕB2F1 and was truncated, replaced by a partially homologous gene, or duplicated elsewhere. Alternatively, there may be a protein analogous in function to Q, not readily identified by DNA sequence, that serves as an antiterminator factor in ϕB2F1. It is also possible that an altogether different antitermination mechanism mediates expression of late genes and toxin in ϕB2F1. The phage repressor of the lytic cycle confers resistance to the host bacterium to challenge by like phages. The repressors, or immunity factors, of ϕB2F1 and 933W differ, as evidenced by the formation of plaques on a 933W lysogen challenged with lysates from B2F1.

The expression of the other toxin allele in B2F1, *stx*$_{2d2}$, was not influenced by bacteriophage induction. The DNA sequence upstream of the *stx*$_{2d2}$ gene was similar to that region upstream of *stx*$_{2d1}$ and shared homology with cryptic phage CP-933O. However, the abrupt divergence of these 5'-flanking regions 1.9 kb upstream of *stx*$_{2d1}$and *stx*$_{2d2}$ suggests that an insertion, deletion, and/or recombination event occurred during the evolution of B2F1 that either provided a mechanism for Stx2d1 phage repression or that disengaged Stx2d2 expression from phage-mediated repression (or both). The sequence downstream of *stx*$_{2d2}$ was most similar in structure and sequence to CP-933V, the cryptic *stx*$_1$-bearing phage of O157:H7. I observed from the DNA sequence that the holin lysis gene homologue downstream of *stx*$_{2d2}$ was truncated. If transcription of the *stx*$_{2d2}$ gene along with the late phage genes were constitutive, then defects in the late genes that prevent the lytic phase, coupled with tight regulation of Stx2d1-phage induction would

likely be necessary to maintain a lysogenized population of *E. coli*. Therefore, I concluded that *stx*$_{2d2}$ is encoded within a defective or cryptic phage.

The sequences flanking *stx*$_{2d1}$ and *stx*$_{2d2}$ showed homology to more than one bacteriophage. The recombinational promiscuity of lambdoid phages both within and among species has been well documented (Hendrix *et al.*, 1999). Moreover, Johansen *et al.* analyzed *stx*$_2$-encoding phages from various O157:H7 strains and described them as mosaics in which the toxin genes and basic lambdoid phage organization are conserved, and these authors also speculated that the heterogeneity of individual phage genes reflects exchanges among a broader gene pool (Johansen *et al.*, 2001). Unkmeier and Schmitt have shown that the chromosomally-encoded variant toxin genes *stx*$_{2c}$ and *stx*$_{2f}$ as well as *stx* in *Shigella dysenteriae* type 1 are flanked with DNA of phage origin and hypothesized that all Stxs are bacteriophage-associated, whether or not they are actually inducible (Unkmeir and Schmidt, 2000). Genomic sequencing has demonstrated that up to 20% of the *E. coli* O157:H7 chromosome is comprised of bacteriophage DNA that could provide many opportunities for intragenomic homologous recombination as well as recombinational exchange with newly acquired and cryptic bacteriophages (Perna *et al.*, 2001). I speculate that a recombinational event occurred that resulted in the duplication of the Stx2d toxin gene in B2F1, and only one of the toxin alleles so duplicated localized within a phage that could be induced.

III. Genes associated with reduced Stx2d2 expression in DH5α, and the effects of comparable mutations on toxin expression in B2F1.

I postulated that expression of Stx2d2, which was uninfluenced by phage induction, is regulated by other bacterial host-derived factors. Wagner *et al.* made isogenic lysogens of *stx*$_2$-converting phages derived from several different clinical isolates. Those investigators observed that the levels of Stx2 produced by the isogenic lysogens differed, even though the host strains were identical. These findings indicated to the authors that phage-specific factors influence the degree of expression of the toxin genes that they encode. However, the same study also showed that the levels of Stx2 produced by the clinical isolates differed in some cases from the toxin levels produced by the corresponding laboratory-derived lysogens. This result suggests to me that bacterial host factors also influence the levels of Stx2 expression, even for strains in which phage regulation is the dominant influence on toxin expression (Wagner *et al.*, 1999). Indeed, RecA is a host factor that has been shown to strongly influence expression of Stxs, especially those that are phage-encoded (Fuchs *et al.*, 1999; Muhldorfer *et al.*, 1996). The search for host factors that influence Stx2d2 expression in this study was based on indirect evidence of a transcriptional activator of Stx2d2 expression in DH5α. Therefore, a transcriptional fusion of the *stx*$_{2d2}$ promoter region with a promoterless *lacZ* gene was constructed and used to screen transposon mutants of DH5α grown on X-gal agar to identify colonies with a white phenotype (promoter "off").

Transposon mutagenesis of DH5α yielded 4 distinct mutations that demonstrated reduced *lacZ* expression from the *stx*$_{2d2}$ promoter::*lacZ* reporter. As predicted from the reporter screening, these DH5α mutants also showed reduced cytotoxicity when transformed with intact *stx*$_{2d2}$ gene clones. The reduction in cytotoxicity seen in the mutants of DH5α was most dramatic (100- to 1000-fold reductions in CD_{50}/ml of culture)

when the mutants were transformed with pSQ545 (stx_{2d2} clone in pKS). Similar results were obtained with Stx2d1- and Stx2-expressing clones borne on high copy number vectors. Mutants transformed with an stx_{2d2} clone in a medium copy number vector (pACYC184) showed a 4- to 5-fold reduction in cytotoxicity. The influence of the mutations appeared to be toxin gene-specific, since expression of unrelated genes (*lacZ* and *tir)* carried on comparable vectors was not substantially reduced in these mutants. The yield of pSQ545 plasmid DNA from the DH5α mutants was about half that seen from wild type DH5α, but the logarithmic reduction in toxin expression from pSQ545 could not be attributed to that minor difference in stx_{2d2} gene copy number alone.

Three of the four genes in DH5α that contained unique transposon insertions were identified. None of these three genes had previously been described as a regulatory gene. One mutation, in *caiD* (Mutant 31), occurred in the carnitine metabolism pathway, just upstream of a gene putatively identified as encoding a transcriptional activator (*caiE*). The proximity of the mini-Tn*5* Km^5 insertion upstream of *caiE* suggested a possible polar effect on that gene. Another insertional mutation (in Mutants 1 and 4) occurred in a cell division-associated gene, "div". Since a homologue of that gene exists on both the K-12 and O157:H7 chromosomes, the direct effect of the "div" gene mutation on the bacterial cell might be less significant than a putative polar effect on the downstream *pdxB* gene (another metabolic, B12 synthesis gene). The third mutation (Mutant 38) occurred in the *ycdU* gene, a locus predicted from its DNA sequence to encode a transmembrane protein. No homologue or function has been identified for *ycdU*.

In a small preliminary *in vivo* study of mice fed DH5α mutants 4 (div gene), 31 (*caiD* gene), and 38 (*ycdU* gene) that had been transformed with pSQ545, I observed

reduced mortality and an extended time to death when compared to the DH5α wild-type similarly transformed. These results suggest that virulence, in addition to *in vitro* cytotoxicity, of the pSQ545-transformed mutants was reduced.

In contrast to the reduction in toxin gene expression seen in DH5α, the B2F1 *caiD* and "div" gene mutants did not differ from wild-type B2F1 in cytotoxicity for Vero cells (I was unable to obtain a resolved *ycdU* mutant in B2F1). Similarly, I saw no attenuation of virulence in mice fed the B2F1 mutant strains compared to wild-type B2F1. One possible explanation for the failure of homologous mutations in B2F1 to show a reduction in toxin expression may be that the magnitude of the influence on toxin expression exerted by the mutants was too small to be observed with one copy of stx_{2d2} in B2F1. Indeed, the greatest reduction in cytotoxicity of the DH5α mutants (2 to 3 logs) compared to wild-type occurred when the mutants were transformed with high copy number toxin clones. Four- to five-fold reductions were observed with medium copy number clones. Since the influence of the DH5α mutations on toxin expression was related to the number of toxin genes provided *in trans*, I hypothesize that in strain B2F1, where one or at most two toxin genes are present, the influence of mutations in *caiD* or "div" on toxin expression is too small to detect.

Another possible reason for the discrepant results on toxin expression in the "div" and *caiD* mutants in a DH5α versus a B2F1 background is that K-12 genes might not exhibit the same properties as their homologues in a virulent clinical strain of *E. coli*, such as B2F1. Although I identified and cloned the *caiD*, "div," and *ycdU* genes from B2F1 by PCR and determined that their sequences were greater than or equal to 97% identical to their K-12 counterparts, I do not know whether they are expressed to the

same extent in B2F1 and K-12. Similarly, I do not know whether these genes serve the same function in these two hosts. Also, the flanking genes may be different in B2F1; hence, the mutations in the B2F1 homologues may not exert the same polar effects as comparable mutations in DH5α. In support of this latter theory are the findings on sequence divergence between the K-12 MG 1655 and the EHEC O157:EDL933 genomes (Perna *et al.*, 2001). Comparison of the genomic sequences of the non-pathogenic K-12 and the pathogenic EHEC *Escherichia coli* shows that these strains share a homologous 4.1 megabase (Mb) backbone. However, the O157:H7 strain contains 1.34 Mb of DNA not found in K-12, and K-12 contains 0.53 Mb of sequence distinct from O157:H7. Although these differences include the virulence determinants of O157:H7 such as toxin genes, and the locus of enterocyte effacement, they also represent biosynthetic and metabolic pathways and open reading frames with no known functions. In addition, these variant sequences are found in segments within the homologous backbone and usually represent multi-gene units. These dissimilar regions have been termed "O-islands" and "K-islands" because they appear to have been acquired in blocks by horizontal transfer, as described for pathogenicity islands. Additionally, like pathogenicity islands, the O- and K-islands are often adjacent to tRNA genes. Many of these strain-specific multigenic islands are comprised of bacteriophage DNA inserted at different points in chromosomes of both the laboratory and wild type strains (Perna *et al.*, 2001). Therefore, there is a large potential for divergence, despite the close homology of specific genes in K-12 and pathogenic *E. coli* such as O157:H7 and, I assume, strain B2F1. The genes that I targeted in DH5α influenced toxin expression in that context with multiple copies of the toxin

gene present. The same genes may be expressed differently and have little influence on toxin expression in the context in which they are found in B2F1.

IV. Unanswered questions

To date, we do not know the position of the Stx2d genes on the B2F1 chromosome or what adjoining genes, other than the bacteriophage genes we have identified, may be present that exert an influence on Stx2d expression. The stx_{2d1} seems to be silent except under conditions that lead to bacteriophage induction. Presumably, stx_{2d1} is tightly regulated by a phage repressor that has not been defined. Also, it is unclear whether Stx2d2 expression is constitutive, repressed (albeit less rigorously than that of Stx2d1), or up-regulated.

V. Summary and conclusions

The studies described here with the individual toxin-producing mutants of B2F1 reveal that Stx2d2 is expressed at levels lethal for mice independent of phage induction, whereas an Stx2d1-producing B2F1 derivative is lethal for mice under bacteriophage-inducing conditions. Although many STEC strains encode more than one toxin type, B2F1 is the first STEC organism described, to our knowledge, where two Stx2 variants are differentially regulated. Furthermore, the isolation of an Stx2d toxin-converting phage demonstrates that this activatable toxin has the potential to be genetically transferred horizontally and reinforces the therapeutic guidelines that quinolone therapy is contraindicated for treatment of *E. coli* O157:H7 infections (Wong *et al.*, 2000; Zhang *et al.*, 2000).

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